NOVEL ANALYTICAL APPROACHES FOR THE DETERMINATION OF LEUCINE ENKEPHALIN AS A MODEL FOR OPIOID PEPTIDES

By
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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1994

To my parents, Charles Larsimont and Kruawan Kanjanasuwan-Larsimont for their unfailing support and encouragement of all my endeavors.

ACKNOWLEDGMENTS

My thanks go to my advisor, Dr. Günther Hochhaus and the members of my supervisory committee, Dr. Hartmut Derendorf, Dr. Paul Klein, Dr. Laszlo Prokai and Dr. Ian Tebbett for their guidance and support during the course of my doctoral research. Special thanks go to Dr. Prokai for the mass spectrometry analysis of hydroxylated leucine enkephalin derivatives. I am also grateful to Dr. Richard Prankerd for his help in the early stages of this work.

I acknowledge the P.D.A. Foundation for Pharmaceutical Sciences, Inc. and Schering-Plough Corporation for partial funding of the research presented in this dissertation.

I would also like to recognize the services provided by the Hybridoma Core and the Protein Chemistry Core of the Interdisciplinary Center for Biotechnology Research at the University of Florida.

There are many others who are too numerous to mention, who have been instrumental in enabling me to complete this work. I hope to be able to thank each of them personally.

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in
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August 1994

Chairman: Günther Hochhaus
Major Department: Pharmaceutics

The focus of this dissertation was the evaluation of novel analytical approaches for opioid peptides by immunoassay or high performance liquid chromatography (HPLC) using leucine enkephalin (LE) as a model peptide.

The proposed immunoassays are based on the high affinity exhibited by avidin for biotin ($K_d=10^{-15}$ mol/l). The successful development of the enzyme-linked immunosorbent assay relied on the formation of a sandwich between anti-LE antibody, a biotinylated LE derivative and avidin, whereas the successful development of the homogeneous fluorescence immunoassay depended on a lack of sandwich formation. The formation of a sandwich was not achieved using any combination of the two anti-LE antibody preparations and several biotinylated LE derivatives tested, and therefore efforts in this direction were abandoned. However, using a polyclonal antibody produced in this laboratory, an N-terminal biotinylated LE derivative without a spacer arm and fluorescein isothiocyanate avidin, an homogeneous fluorescence immunoassay for LE was developed which was operational in a narrow concentration range ($1*10^{-9}$ to $1*10^{-8}$ moles LE/ml).

Two HPLC assays for opioid peptides were evaluated. One was based on tyrosine-specific pre-column hydroxylation using tyrosinase, specific sample clean-up using a boronate gel and HPLC with electrochemical detection. The other involved tyrosine-specific pre-column hydroxylation using tyrosinase followed by fluorogenic derivatization using 1,2-diamino-1,2-diphenylethane and HPLC with fluorescence detection. These assays yielded limits of detection for LE of 170 fmol/inj and 500 fmol/inj respectively in buffer samples and 360 fmol/inj and 500 fmol/inj respectively in spiked cerebrospinal fluid samples.

Using electrospray ionization mass spectrometry, the structure of the products of the reaction between LE and tyrosinase were found to be monohydroxylated LE ([HO-Tyr^1]-LE) and dihydroxylated LE ([(HO)$_2$-Tyr^1]-LE). Compared to LE, the affinity of [HO-Tyr^1]-LE to both μ and δ opioid receptor sites in rat brain homogenate was found to be lower by a factor of about 20. Since enkephalins and tyrosinase have been found to co-exist in vivo, we speculate that tyrosinase may play role in the metabolic pathway of these compounds.

CHAPTER 1
INTRODUCTION

Endogenous Opioid Peptides

There exists three separate families of natural or endogenous opioid peptides: enkephalins, endorphins and dynorphins. These three families are derived from three different prohormones, proenkephalin, pro-opiomelanocortin and prodynorphin, respectively and are coded by messenger RNAs from three separate genes (Figure 1.1) [Pleuvry 1991].

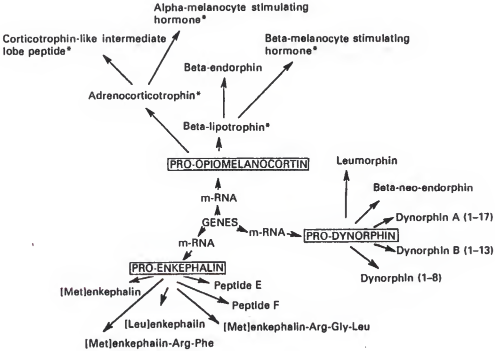


Figure 1.1. Opioid peptides and their precursors. (* Denotes no opioid receptor activity) [Pleuvry 1991]

In the proenkephalin family, proenkephalin A is the precursor of the pentapeptides methionine enkephalin (ME, Tyr-Gly-Gly-Phe-Met) and leucine enkephalin (LE, Tyr-Gly-Gly-Phe-Leu) which were the first opioid peptides to be characterized by Hughes and coworkers in 1975 (Figure 1.2) [Hughes et al. 1975]. Proenkephalin A has been shown to contain ME and LE in a fixed ratio of six ME sequences to one LE sequence. Proenkephalin-expressing cells are widespread throughout the brain and spinal cord as well as in more peripheral sites such as the adrenal medulla and the gastrointestinal tract.

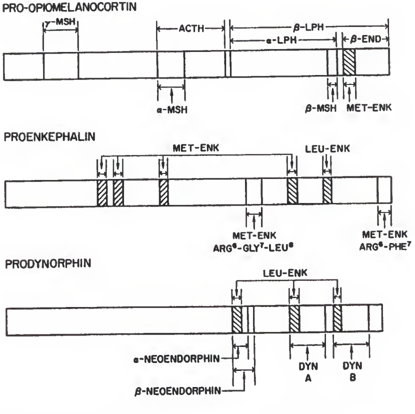


Figure 1.2. Schematic representation of the structures of opioid peptide precursors [Jaffe and Martin 1990].

Pro-opiomelanocortin (POMC) is the precursor of the opioid peptide β-endorphin as well as the non-opioid hormones adrenocorticotrophic hormone (ACTH) and α- and βmelanocyte simulating hormone (MSH, Figure 1.2). The term endorphin applies to opioid peptides derived from POMC. POMC is synthesized in the pituitary but is also present in the hypothalamus and in the periphery. The ME amino acid sequence is present at the N-terminal end of β-endorphin.

The dynorphins as well as β-neoendorphin and leumorphin are derived from prodynorphin which is also known as proenkephalin B. Prodynorphin contains LE sequences but no ME sequences and is synthesized throughout the central nervous system (Figure 1.2).

When the opioid peptide precursors are processed to give the various different opioid peptides, the N-terminal end of the molecules is highly conserved with tyrosine in the 1 position. However, tyrosine is often absent in this position in non-related peptides. This phenomenon is exploited in two of the analytical approaches described in this dissertation.

Opioid Receptors

Opioid receptors are widely distributed throughout the central nervous system of all vertebrates and have also been found in a number of peripheral tissues including the intestinal tract, the adrenal and pituitary glands and the vasa diferentia of several species [Simon 1984].

At present, three classes of opioid receptors are firmly recognized, predominantly on the basis of in vivo studies of opioid action (agonists and antagonists), in vitro bioassays and binding experiments with selective ligands [Simonds 1988]. These three classes of receptors are defined as mu, kappa and delta. The existence of sigma and epsilon receptors has also been postulated; however, there is now some doubt as to whether the sigma receptor is truly "opioid" in nature and epsilon receptors have not been detected with any degree of certainty in any tissue except rat vas deferens. The cloning of opioid receptors using recombinant DNA techniques can be expected to yield information about the differences in the primary amino acid sequence of mu, delta and kappa receptors, and may also uncover new opioid receptor subtypes thus elucidating the structural features responsible for receptor specificity.

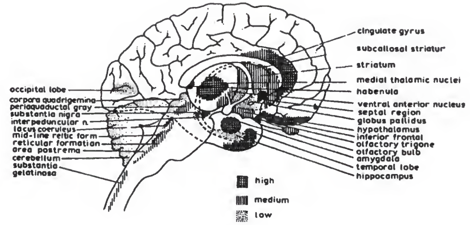


Figure 1.3. Distribution of opioid receptors in the human brain [Simon 1984].

No selective endogenous ligand has yet been isolated for the mu receptor as opioid peptides from all three families bind to this receptor. It has therefore been suggested that the mu receptor may be a "universal" opioid receptor [Akil et al. 1988]. Mu opioid receptor sites are widespread but are found in particular in the regions of the brain associated with pain regulation and sensorimotor integration [Mansour et al. 1988]. It has been proposed that mu receptors may be subdivided into high affinity sites (mu_1) which are thought to mediate supraspinal analgesia and a low affinity sites (mu_2) which are thought to be responsible for respiratory depression and gastrointestinal effects [Pasternak 1982].

Leucine enkephalin and other derivatives of proenkephalin A interact with the delta receptor, although not selectively. Opiate alkaloids, on the other hand, have low affinity for this receptor. Delta receptors are less widespread than mu receptors but are concentrated in neural areas involved with olfaction and motor integration and have been implicated in pain pathways [Mansour et al. 1988].

The derivatives of prodynorphin have selectivity for kappa receptors. Although LE is selective for the delta receptor, as the molecule is lengthened, its preference for the delta receptor is reduced and its affinity for the kappa receptor increases. Kappa receptors are found predominantly in brain areas associated with pain perception and the regulation of water balance and food intake [Mansour et al. 1988].

Physiology and Pharmacology

The binding of an opioid to its receptor triggers a number of complex processes which occur before leading to the ultimate opioid effect. Opioid effects are believed to mediated through guanine nucleotide regulatory proteins (G proteins) which are involved in signal transduction to a variety of effector systems including adenylate cyclase, phospholipidase C and ion channels. Presently, adenylate cyclase inhibition is the best characterized opioid effect mediated by G proteins [Simon 1984].

Adenylate cyclase is an enzyme that synthesizes cyclic AMP from ATP so that it can then go on to act as a "second messenger" in a number of biochemical systems. G-proteins cause adenylate cyclase to convert from the active form of the enzyme which is coupled to GTP to the inactive form which is coupled to GDP and vice versa. Therefore, a compound that stimulates adenylate cyclase production would do so through the interaction of its receptor with a G-protein that converts adenylate cyclase to the active GTP-coupled form, whereas inhibition of adenylate cyclase, as by opioids for example, would be mediated through a G-protein that favors the formation of inactive GDP-coupled adenylate cyclase (Figure 1.4).

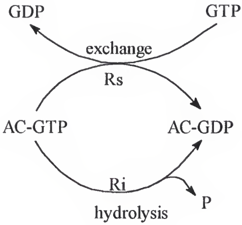


Figure 1.4. Scheme for receptor mediated stimulation and inhibition of adenylate cyclase. Rs - receptors which produce stimulation of adenylate cyclase on binding, Ri - receptors which produce inhibition of adenylate cyclase on binding, AC - adenylate cyclase [Simon 1984].

Opioid peptides are distributed widely throughout the central and peripheral nervous system, suggesting that these compounds play a part in a variety of physiological functions [Olson et al. 1991]. The physiological roles of endogenous opioid peptides have been attributed largely on the basis of effects seen on administration of the opioid antagonist naloxone. These effects are assumed to be the result of opioid receptor blockade and are widely accepted as indirect evidence for endogenous opioid involvement in the physiological function under observation.

One of the most important functions of opioid peptides is in pain modulation. The mu receptor is the opiate receptor most associated with pain relief, although delta and kappa receptor agonists also have analgesic properties. It is thought that under some conditions, mu and delta receptors are functionally coupled as delta agonists given in sub-analgesic doses have been found to either potentiate or inhibit the analgesic effects of morphine (a mu agonist) at different sites. It has therefore been postulated that mu and delta receptors may exist either separately or in a complexed form [Rapaka and Porreca 1991]. Acupuncture analgesia is also thought to be mediated by endogenous opioid peptides [Clement-Jones and Rees 1982].

Animal studies have indicated that endogenous opioid peptides play a role in the development of opiate dependence, a side effect common to opiate analgesics. This is a syndrome whereby distress is caused upon withdrawal of an opiate following chronic administration. It has been hypothesized that chronic narcotic abuse leads to the suppression of endogenous opioid production through a negative feedback mechanism so that sudden withdrawal of the narcotic leads to a deficiency in endogenous opioids which causes the classical physical withdrawal symptoms [Clement-Jones and Besser 1983]. Recently, Wang et al. [Wang et al. 1994] have proposed that stimulation by an opiate agonist causes gradual constitutive mu receptor activation so that an agonist is no longer required for signal transduction, and a dependent state is established, consisting of an upregulated cAMP system, counterbalanced by constitutively active mu receptors. In this model, opiate tolerance results from fewer mu receptors remaining activatable by agonists and the enhanced activity of the cAMP system. In other words, in this scenario dependence occurs because an upregulated cAMP system is established which needs to be counterbalanced by opiate agonist activity and tolerance occurs because fewer mu receptors can now be activated by opiate agonists.

A lower degree of dependence is seen with agonists at delta receptors than with agonists at mu receptors. As mentioned above, the action of mu receptor agonists can be modulated by the co-administration of delta receptor agonists so that the potency and efficacy of analgesia is increased without a corresponding increase in side effects such as physical dependency, respiratory depression and gastrointestinal effects. This effect could be exploited to allow for the use of mu agonists of lower efficacy and increased safety while still providing adequate pain relief without the risk of side effects. Eventually, a delta agonist may be developed which is able to provide effective pain relief without side effects [Rapaka and Porreca 1991].

Opioid peptides play a part in the regulation of the immune system, particularly during periods of stress, as they modulate the functions of a number of cell types involved in the immune response [Murgo et al. 1986]. Generally, endogenous opioid peptides are immunostimulant as they enhance T-cell function and stimulate phagocyte function, thus increasing resistance to infection. There is also evidence that suggests a role for endogenous opioids in the growth and development of lymphoid tissue [Plotnikoff et al. 1985].

Opioid peptides are thought to depress the responsiveness of the chemosensors to carbon dioxide and may therefore play a physiological role in the control of respiration [McQueen 1983]. This applies in particular to neonates and to adults in stressful situations [Pleuvry 1991].

Opioid peptides may also be involved in blood pressure regulation as they are present in nerve fibers in areas of the brain stem responsible for the regulation of blood pressure and the secretion of vasopressin. Biochemical evidence suggests that opioid peptides interact with neurohormones to regulate blood pressure. Opioid peptides have been implicated in the dramatic changes in blood pressure which occur during sleep and in hypotension due to various states of shock [Rubin 1984]. It has also been suggested that endogenous opioid peptides may be involved in the pathogenesis of hypertension [Szilagyi 1989].

The presence of opioid peptides within limbic structures suggests their involvement in the regulation of mood and behavior. Endogenous opioid peptides are also known to interact with the central catecholamines implicated in psychiatric disease. However, the results of studies carried out to determine the role of opioid peptides in psychiatric disease have been contradictory [Clement-Jones and Besser 1983, Koob and Bloom 1983], and therefore no conclusions can be drawn at present.

The very high concentrations of opioid peptides present in the hypothalamus suggests a role for these substances in neuroendocrine regulation. Opioid peptides may control the secretion of anterior pituitary hormones by modifying the release of hypothalamic anterior pituitary regulating substances. This may be the mechanism by which opioid peptides cause an increase in the secretion of prolactin, growth hormone and thyrotrophin and inhibit the release of luteinizing hormone, follicle stimulating hormone, adrenocorticotrophic hormone and beta- and gamma-lipotropin [Clement-Jones and Besser 1983, Grossman and Rees 1983]. Further possible functions of opioid peptides can be found in Table 1.1.

Table 1.1. Possible physiological functions of endogenous opioid peptides [Imura et al. 1985].

1. Defense against noxious stimuli
    - Activation of the pituitary-adrenocortical axis
    - Regulation of the sympatho-adrenal system
    - Inhibition of pain perception

2. Modulation of vegetative nervous system
    - Cardiovascular and respiratory system
    - Gastrointestinal tract and pancreas
    - Genito-urinary tract

3. Modulation of neuroendocrine function
    - Anterior and posterior pituitary hormones
    - Gastrointestinal and pancreatic hormones
    - Catecholamines

4. Behavioral action
    - Mood and locomotor activity
    - Food and water intake
    - Sexual behavior

The design of opioid peptides as therapeutic agents has several advantages. Firstly, these substances are endogenous so that their metabolites are likely to be non-toxic and not to cause renal or hepatic damage, depending on the doses administered. Secondly, a large number of analogs can be synthesized from a few basic amino acid building blocks as synthesis has been simplified and automated and simple modifications can be used to develop different analogs with desirable biological activities. Opioid peptides are unable to cross the placental barrier as they are subject to placental enzymatic deactivation and would therefore be ideal for obstetric use [Rapaka 1986, Rapaka and Porreca 1991].

At present, the therapeutic development of opioid peptides is focused on their potential as analgesic agents and in the treatment of opiate addiction. Research efforts are directed towards the design of analgesic peptides which can be administered orally, have a long duration of action and reduced potential for dependence and abuse. Peptides of interest include enkephalins, endorphins and related opioid peptides.

Opioid peptides are easily degraded by aminopeptidases which hydrolyze the Tyr^1-Gly^2 bond, carboxypeptidases which cause cleavage at the C-terminal end of the molecule, relatively non-specific enzymes such as trypsin and angiotensin converting enzyme (ACE) and more specific enzymes such as enkephalinases which hydrolyze the Gly^3-Phe^4 in enkephalins. This has led to the suggestion that enkephalin degrading enzymes be used as an alternative therapeutic approach [Rapaka 1986, Rapaka and Porreca 1991]. Examples of these enzyme inhibitors include bestatin, thiorphan and captopril which inhibit aminopeptidase, enkephalinase and ACE, respectively. These inhibitors prolong the duration of action of endogenously released enkephalins and it is therefore hoped that they are free of the side effects produced by narcotic analgesics. The clinical use of these substances may however be limited due to their limited bioavailability.

Another approach which has been used to increase both the stability and the selectivity of opioid peptides is the introduction of synthetic modifications to the molecule [Shimohigashi 1986]. For example, stability can be increased by substituting the corresponding D-amino acid for the naturally occurring L-amino acid in the peptide molecule. As peptides are conformationally labile, the selectivity of opioid peptides has been increased by stabilizing the peptide molecule in a conformation which prefers the desired receptor. This can be achieved through the incorporation of conformational restrictions such as the introduction of unnatural bulky synthetic amino acids (e.g./ penicillamine residues) or the cyclization of the peptide chain. Highly selective opioid peptide analogs such as [D-Pen^2, D-Pen^5]-enkephalin which is selective for the delta receptor [Mosberg et al. 1983] and [Tyr-D-Ala-Gly-MePhe-NH$(CH_2)_2$OH] which is selective for the mu receptor [Handa et al. 1981] have been developed using these techniques.

Further research involving opioid peptides and their receptors is of importance in elucidating the precise physiological roles of these entities, particularly in the areas of pain and immunomodulatory pathways.

Rationale

As discussed above, a considerable body research has been focused on the development of opioid peptides as therapeutic agents. The low physiological concentrations of endogenous opioid peptides and those to be expected for therapeutically administered derivatives necessitate the development of specific and ultra-sensitive analytical methods, in the fmol per ml range, for these entities in biological fluids to support clinical studies. The need for new analytical approaches for the measurement of opioid peptides has been stressed by the National Institute on Drug Abuse [Rapaka 1986].

Current analytical methods for opioid peptides are summarized in Table 1.2 and are reviewed in the following pages.

Immunological Methods

At present, for the most part, opioid peptides are analyzed by radioimmunoassay (RIA) [Sato 1984, Venn 1987]. Although immunoassays are highly sensitive and reproducible, they are hampered by several disadvantages. Firstly, an antiserum to the analyte of interest has to be raised, which involves lengthy incubation times. Secondly, immunoassays suffer from low selectivity due to the cross-reactivity of the antiserum to structurally similar compounds. To overcome the problem of cross-reactivity, high performance liquid chromatography (HPLC)-immunoassay methods have been developed whereby collected eluted fractions from an HPLC system are analyzed by RIA [Defrutos and Regnier 1993, McDermott et al. 1981]. However, HPLC-immunoassay methods are time consuming, labor intensive and chromatographic solutions must be volatile or compatible with the immunoassay. Radioimmunoassays also have the added disadvantage that the use of radioactive isotopes as tracers calls for special considerations in the handling of the assays and the disposal of the radioactive waste produced.

Non-radiation immunological techniques have not been exploited to a great extent for opioid peptides. An enzyme-linked immunosorbent assay (ELISA) has been described for LE and ME [Zamboni et al. 1983] but its limits of detection are only in the 1 pmol per assay range. Only four enzyme immunoassays with high sensitivity have been developed for β-endorphin and dynorphin [Hochhaus and Hu 1990, Hochhaus and Sadee 1988, Kuhling et al. 1989, Sarma et al. 1986]. These assays have sensitivities ranging from 0.3 to 3.2 femtomole per assay.

The immunoassays developed by Hochhaus and Sadee [Hochhaus and Sadee 1988] and Hochhaus and Hu [Hochhaus and Hu 1990] are ELISAs based on the avidin-biotin system whereby the peptide of interest in the sample or standard and its biotinylated derivative compete for antibody binding sites. The antibody-bound biotinylated species is subsequently detected by enzymatic detection through the use of an avidin-enzyme complex. For this dissertation, an attempt was made at the development of an extremely sensitive avidin-biotin based ELISA for enkephalins, using LE as a model peptide, to compliment the existing ELISA tests for β-endorphin and dynorphin. If this type of assay can be developed for LE, it can be expected to be also applicable to ME.

In addition, for this dissertation, an attempt was also made at the development of an homogenous or non-separation immunoassay for opioid peptides. Homogeneous immunoassays differ from traditional immunoassays in that the labor intensive separation of the bound and free fraction of the analyte (e.g./ by washing, precipitation or adsorbance) is not necessary prior to quantitation as the property being measured is characteristic of either the bound or the free analyte or label.

The immunological techniques proposed in this dissertation have the disadvantage of low specificity, but they were intended for use as "immunological HPLC detectors" in the hope that they would provide fast, ultra-sensitive assays which were highly suitable for processing large numbers of samples such as HPLC fractions.

Table 1.2. Summary of analytical methods for opioid peptides.

Reference	Method	Sensitivity	Analyte	Comments
Fleming and Reynolds 1988	HPLC-ED	1 pmol/inj	enkephalin	tedious sample clean up, high oxidation potential
Kim et al. 1989	HPLC-ED	1 pmol/inj	enkephalin	tedious sample clean up, high oxidation potential
Shibanoki et al. 1990	HPLC-ED	550 fmol/inj	enkephalin	tedious sample clean up, high oxidation potential
Monger and Olliff 1992	HPLC-ED	75 fmol/ ml plasma	β-endorphin	tedious sample clean up, high oxidation potential
Muck and Henion 1989	HPLC-MS	100 fmol/inj	dynorphin	microbore LC system
Mifune et al. 1989	HPLC-FL	100 fmol/inj	enkephalin	complicated column switching
Nakano et al. 1987	HPLC-FL	140 fmol/inj	enkephalin	harsh reaction conditions
Kai et al. 1988	HPLC-FL	500 fmol/inj	enkephalin	harsh reaction conditions
van den Beld et al. 1990	HPLC-FL	50 fmol/inj	β-endorphin	laser induced fluorescence
Dave et al. 1992	HPLC-FL	36 fmol/inj	LE	microbore LC system
Hochhaus and Sadee 1988	ELISA	<1 fmol/assay	β-endorphin	
Hochhaus and Hu 1990	ELISA	1 fmol/assay	dynorphin	
Kuhling et al. 1989	ELISA	3 fmol/assay	β-endorphin	
de Ceballos et al. 1991	HPLC-RIA	1.5 fmol/assay	LE, ME	
Maidment et al. 1989	RIA	<1 fmol/assay	ME	

HPLC-ED = high performance liquid chromatography with electrochemical detection, HPLC-FL = high performance liquid chromatography with fluorescence detection, HPLC-MS = high performance liquid chromatography with mass spectrometry, ELISA = enzyme-linked immunosorbent assay.

Instrumental Approaches

Other methods which have been used in the analysis of opioid peptides include HPLC combined with electrochemical detection (HPLC-ED), fluorescence detection (HPLC-FL) or mass spectrometry (HPLC-MS).

Electrochemical detection

HPLC in conjunction with electrochemical detection is an analytical method which offers several advantages. It provides selectivity, as only those compounds which are oxidizable or reducible at the applied potential will be detected. Multiple electrode detectors can be used to pre-oxidize contaminants in the mobile phase and the sample prior to detection of the analyte of interest, thus increasing sensitivity by improving signal to noise ratios. The method is versatile and the cost of instrumentation and reagents is relatively low. In electrochemical detection, peak current ratios are obtained from the ratio of the peak heights obtained (i.e. current generated) when two different voltages are applied to the same amount of sample. These peak current ratios are characteristic for each compound, much like absorbance ratios in ultraviolet detection, and therefore qualitative information about the analyte can be derived from them.

A review of the literature reveals that, to date, HPLC-ED methods for opioid peptides are less sensitive than other methods such as RIA [Fleming and Reynolds 1988, Kim et al. 1989, Mousa and Couri 1983]. This may be attributed to the fact that the high potentials necessary to detect opioid peptides using these methods (+0.9-1.25 V compared to +0.3-0.4 V used in HPLC-ED assays for catechols with sensitivities in the fmol/inj range [Higa et al 1977, Koike et al. 1982]) compromise sensitivity as background current and baseline noise are increased significantly. Selectivity is also compromised as more compounds are oxidized at these high potentials, thus necessitating extensive sample clean up.

In the HPLC-ED assay for LE described in this dissertation, the enzymatic derivatization used increased HPLC-ED selectivity and sensitivity by pre-column *o*-hydroxylation of the highly conserved N-terminal tyrosine groups of the peptide resulting in easily oxidizable derivatives.

Fluorescence detection

Pre-column fluorescence derivatization of analytes can be achieved by fluorophoric labeling using a fluorescent precursor, or by fluorogenic derivatization using a non-fluorescent precursor. Fluorogenic derivatization is usually preferred as fluorophoric labeling often requires excess fluorescent reagent and subsequent extensive clean up to minimize background interference.

Fluorogenic derivatization reactions have been carried out by using fluorogenic reagents such as *o*-phthalaldehyde [Roth 1971], fluorescamine [Udenfriend et al. 1972] and naphthalene-2,3-dialdehyde in the presence of cyanide [Lunte and Wong 1989, Mifune et al. 1989]. However, these procedures derivatize N-terminal amino groups and consequently are not specific for any particular peptide so that subsequent chromatographic procedures are often extremely complex (e.g./ multidimensional HPLC systems using column switching) to allow for the selective determination of opioid peptides as the derivatives of other peptides may interfere with the signal.

A derivatization reaction which is specific for tyrosine groups such as the one described in this dissertation could be expected to increase selectivity in the determination of opioid peptides as tyrosine is highly conserved in the 1 position of these compounds but is often missing in unrelated peptides. Tyrosine specific HPLC methods with fluorescence detection such as those using 1,2-diamino-4,5-dimethoxybenzene [Ishida et al. 1986, Kai et al. 1988] and hydroxylamine, cobalt (II) ion and borate [Nakano et al. 1987, Zhang et al. 1991] do exist for peptide analysis. Although these methods have allowed the determination of opioid peptides with sensitivities of 100-500 femtomole per injection, the harsh reaction conditions of these derivatizations are expected to lead to diminished recoveries and reduced assay reproducibility of the fragile peptide analytes.

Mass spectrometry

HPLC with mass spectrometry offers the highest level of molecular specificity compared to other analytical methods and is the only method with which unambiguous confirmation of the structure of the target peptide can be achieved. However, MS is costly and requires specialized instrumentation and therefore, it is an impractical method for the average analytical laboratory. The analysis of peptides by mass spectrometry has been facilitated in recent years by the advent of several “soft” ionization and sample introduction techniques such as fast atom bombardment, matrix-assisted laser desorption, electrospray and ionspray mass spectrometry (see Arnott et al. 1993, Biemann 1992 and Carr 1990 for reviews) which allow the production of intact molecular ions from these fragile species. Another advantage of these techniques is that they are often compatible with on-line microbore HPLC. A microbore HPLC-ionspray MS method has allowed the determination of enkephalins in the 100 fmol/inj range [Muck and Henion 1989]. Levels as low as 5 fmol of β-endorphin and 1 pmol of ME have been quantified by electrospray mass spectrometry with off-line HPLC [Dass and Kusmierz 1991] and pmol amounts of ME have been detected by fast atom bombardment mass spectrometry with off-line HPLC [Kusmierz and Sumrada 1990].

Objectives

Appropriate analytical methodology for opioid peptides is required for use in clinical, pharmacokinetic and formulation studies as well as in physiological studies to allow the successful development of these entities as therapeutic agents and the continuation of research to elucidate further the physiological role of these compounds. The need for accurate, specific, sensitive and reproducible analytical methods for opioid peptides has been stressed by representatives of the National Institute on Drug Abuse [Rapaka 1986]. However, to date no analytical procedure has emerged which adequately meets all of these criteria.

Therefore, the focus of the work carried out for this dissertation has been the evaluation of several novel analytical approaches for the determination of leucine enkephalin (LE, Figure 1.5) as a model for opioid peptides. Leucine enkephalin was chosen as a model peptide as it contains the same initial sequence common to all opioid peptides.

The first approach evaluated was a non-homogeneous enzyme-linked immunosorbent assay (ELISA) which employed the same strategy as ELISAs which have been previously developed for dynorphin [Hochhaus and Hu 1990] and β-endorphin [Hochhaus and Sadee 1988]. This assay was based on the avidin-biotin system whereby avidin exhibits an extremely high affinity for biotin and biotinylated species. Here, LE and biotinylated LE derivative compete for antibody binding sites and subsequently, on separation of the antibody-bound and free fractions, the antibody-bound biotinylated species is detected through the use of an avidin-enzyme complex. The success of this type of assay depends on the formation of a "sandwich" between the antibody, the biotinylated LE derivative and enzyme-labeled avidin.

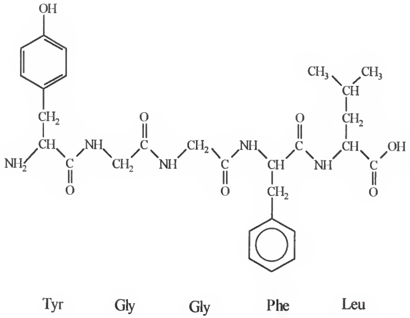


Figure 1.5. Leucine enkephalin.

The second approach was an homogeneous or separation-free fluorescence immunoassay which also made use of the avidin-biotin system, and was based on an observation by Al-Hakiem and co-workers [Al Hakiem et al. 1981] that the binding of biotin to fluorescence-labeled avidin produced an increase in fluorescence intensity. In contrast to the ELISA described above, this approach relied on a lack of sandwich formation between antibody, biotinylated LE derivative and fluorescence-labeled avidin, as the success of this approach depended on the inability of antibody-bound biotinylated LE to interact with fluorescence-labeled avidin and produce an increase in fluorescence intensity. Therefore, here LE and biotinylated LE compete for antibody binding sites and after equilibrium has been achieved, free biotinylated LE is detected through the increase in fluorescence intensity produced on addition of fluorescence-labeled avidin.

Two high-performance liquid chromatography (HPLC) assays were also evaluated, both making use of tyrosine-specific pre-column derivatization using the enzyme tyrosinase. In the high performance liquid chromatography assay with electrochemical detection (HPLC-ED), specific hydroxylation of the tyrosine group in the 1 position of LE, which is highly conserved in all opioid peptides, presents two analytical advantages. Firstly, the derivatization results in the formation of a catechol which is amenable to specific clean-up using boronate gels, and secondly, this catechol is more easily oxidizable than the parent peptide, thus facilitating electrochemical detection. In the high performance liquid chromatography assay with fluorescence detection (HPLC-FL), enzymatic derivatization by tyrosinase renders our peptide amenable to fluorogenic derivatization using 1,2-diamino-1,2-diphenylethane.

In addition, having established in the enzymatic derivatization for the HPLC assays that products are formed from the reaction between LE and tyrosinase, the identity of these products was determined by mass spectrometry. Furthermore, since tyrosinase and enkephalins have been found to co-exist in vivo [Merchenthaler 1993, Sesack and Pickel 1992, Zhuo et al. 1992], the biological activity in rat brain homogenate of the major product of the reaction between these two entities was investigated.

CHAPTER 2
APPROACHES TO THE DEVELOPMENT OF AN IMMUNOASSAY FOR LEUCINE ENKEPHALIN

Introduction

Immunoassay is an analytical method which exploits the binding of a ligand (antigen) to specific sites on an antibody. In most cases (e.g./ for radioimmunoassays), labeled ligand competes with unlabeled ligand (analyte) for antibody sites and the extent of binding of the labeled ligand is determined through the measurement of some physical or chemical property associated with the label. A standard calibration curve of the signal produced by the label with respect to the concentration of analyte present can then be constructed, thus allowing the estimation of unknown ligand concentrations from this curve.

Traditionally, immunoassays have involved the use of radioisotopes as labels. Although radioimmunoassays (RIA) have the advantages of being highly sensitive and invulnerable to environmental interferences (e.g./ by components of the assay), their use is accompanied by several disadvantages. These disadvantages include the emotive bias against the use of radioisotopes, the costly disposal of waste, special requirements for assay handling and training of staff, the lack of stability of radioisotopes and the consequent limited shelf-life of reagents [Gosling 1990]. Therefore, there has been considerable interest in developing non-isotopic labels, such as enzyme labels and luminescent labels for use in immunoassay [Kricka 1993, Porstmann and Kiessig 1992, Schulman et al. 1990].

Immunoassays can be divided into those methods which require separation of the antibody-bound and free fractions prior to quantitation, and those in which the signal being measured is a function of antibody binding and therefore do not require separation of antibody-bound and free fractions prior to quantitation. These assays are referred to as heterogeneous and homogeneous immunoassays, respectively. The development of homogeneous immunoassays was prompted by the fact that the separation step in heterogeneous immunoassays is labor intensive, complicates automation and introduces inaccuracy and error into the assay as the equilibrium which exists between the bound and free fraction in the sample is disturbed. Enzyme- and luminescence- labeling techniques are used in the majority of the homogeneous immunoassays developed thus far [Coty et al. 1992, Garcia et al. 1993, Jenkins 1992]; however, other approaches involving the use of liposomes [Bowden et al. 1986, Ho and Huang 1985, Umeda et al. 1986], bilayer membranes [Ihara et al. 1988] and reversed micellar systems [Kabanov et al. 1989] have also been investigated. Although homogeneous immunoassays are convenient and relatively simple to perform, to date, they have suffered from lack of sensitivity compared to heterogeneous immunoassays, due in particular to interference with endpoint determination by components of biological samples [Jenkins 1992].

As mentioned in Chapter 1, one of the major disadvantages of immunoassays in general is their relative lack of specificity due to cross-reactivity of the antibody in use to structurally similar compounds. In order to circumvent this problem, analytical methods have been developed whereby structurally similar analytes are first separated by high-performance liquid chromatography (HPLC) prior to quantitation by immunoassay. To this end, the goal in the development of the immunoassays proposed in this dissertation was the design of rapid and convenient assays for opioid peptides with "detector-like" properties, intended for the determination of opioid peptide concentrations in HPLC fractions. These assays should therefore allow the sensitive and straightforward analysis of opioid peptides, be practicable on a large scale and easily amenable to intensive automation.

Two different approaches to the development of an immunoassay for opioid peptides were conceived, using leucine enkephalin (LE) as a model peptide. Both of these approaches were based on the exploitation of the extremely high affinity exhibited by avidin for biotin ($K_a=10^{15}$ l/mol). Each avidin molecule has four high affinity binding sites for biotin so that the central strategy of these approaches lies in the knowledge that when avidin is conjugated to an enzyme or fluorescent label, it will still bind to biotin or biotinylated species [Wilchek and Bayer 1988].

The first approach, an enzyme-linked immunosorbent assay (ELISA) for LE is similar to assays previously developed for dynorphin [Hochhaus and Hu 1990] and β-endorphin [Hochhaus and Sadee 1988] which have sensitivities in the lower fmol/assay range. The development of a similar assay for LE would have provided a battery of enzyme immunoassays for opioid peptides based on identical principles. This assay involves competition between LE in the sample or standard and biotinylated LE derivative for immobilized antibody binding sites. The antibody-bound biotinylated species is subsequently detected through the use of an avidin-enzyme complex (Figure 2.1). The successful design of this type of assay for LE depends on the synthesis of a suitable biotinylated LE derivative that will allow the formation of a "sandwich" between the antibody, the biotinylated LE derivative and the enzyme-labeled avidin, thus enabling quantification upon addition of the enzyme substrate.

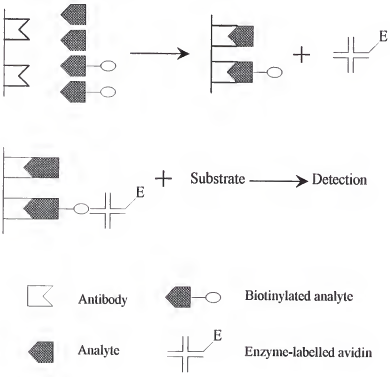


Figure 2.1. Avidin-biotin based ELISA

The second approach, which is an homogeneous or separation-free fluorescence immunoassay is based on an observation by Al-Hakiem et al. [Al-Hakiem et al. 1981] that the binding of biotin to fluorescein-labeled avidin leads to an increase in fluorescence intensity. This phenomenon is exploited in an attempt to develop a new class of homogeneous immunoassay which depends on the inability of antibody-bound biotinylated

LE to interact with fluorescein-labeled avidin. Therefore, in this assay, biotinylated LE and LE in the sample or standard compete for antibody binding sites and after equilibrium has been achieved, fluorescence-labeled avidin, which acts as a "detector" molecule, is added. Unbound biotinylated LE is determined by the increase of fluorescence intensity due to the interaction of the unbound biotinylated LE and the fluorescence-labeled avidin (Figure 2.2). In contrast to the ELISA described above, the success of this assay depends on the synthesis of a biotinylated LE derivative which will not allow fluorescence-labeled avidin to bind to antibody-bound biotinylated LE (i.e. lack of "sandwich" formation).

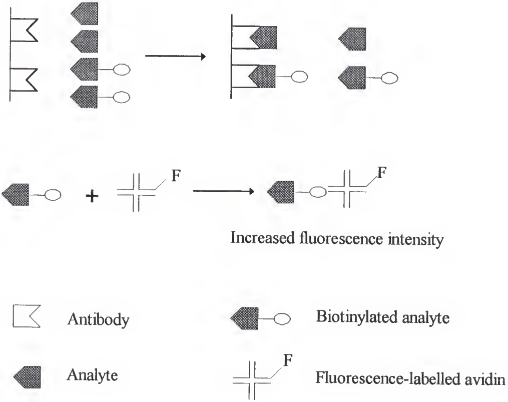

F
+
F
Increased fluorescence intensity
Antibody
Biotinylated analyte
Analyte
F
Fluorescence-labelled avidin

Figure 2.2. Avidin-biotin-based homogeneous fluorescence immunoassay

Materials

Leucine enkephalin, leucine enkephalin-Lys^6, Freund's complete adjuvant, Freund's incomplete adjuvant, bovine serum albumin, porcine thyroglobulin, glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, sodium acetate, activated charcoal, dextran (average molecular weight 70,800), avidin, N-hydroxysuccinimidobiotin, biotinamidocaproate N-hydroxysuccinimide ester, triethanolamine, 2-4'-hydroxyazobenzene-benzoic acid, di-methylsulfoxide and fluorescein isothiocyanate avidin were obtained from Sigma Chemical Company, St. Louis, MO, USA. Methanol, acetonitrile, trifluoroacetic acid, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, potassium dihydrogen phosphate, dipotassium hydrogen phosphate and potassium chloride were purchased from Fisher Scientific, Pittsburgh, PA, USA. [tyrosyl-3,5-^{3}H(N)]-leucine enkephalin was procured from NEN Research Products, Dupont Company, Wilmington, DE, USA. Cytoscint scintillation cocktail was obtained from ICN Biomedicals Inc., Irvine, CA, USA and anti-LE antiserum was purchased from Peninsula Laboratories Inc., Belmont, CA, USA.

Methods

Antibody Production

Leucine enkephalin (LE) was conjugated to either porcine thyroglobulin or bovine serum albumin (BSA) by reaction with either glutaraldehyde or 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC).

For the glutaraldehyde method (G. Adamus, personal communication), a 25 % v/v stock solution of glutaraldehyde was freshly prepared on ice and diluted 65-fold in 0.1 M sodium phosphate buffer pH 7. Equal amounts of LE and protein carrier (BSA or porcine thyroglobulin) were weighed out and dissolved in 0.1 M sodium phosphate buffer pH 7 to give a solution containing 1 mg/ml each of LE and protein carrier. One hundred and twenty four microliters of the final dilution of glutaraldehyde were added for each milliliter of protein-peptide solution and the reaction was allowed to proceed at room temperature overnight with constant stirring. The conjugate was then dialyzed against deionized water for 24 hours using pre-hydrated Spectrapor cellulose ester membranes with molecular weight cut off of 15,000 (Spectrum Medical Industries Inc., Los Angeles, CA, USA). After dialysis, aliquots of the conjugates were stored at -20°C prior to lyophilization.

For the EDC method [Harlow and Lane 1988], a 1 mg/ml solution of LE was prepared in water and EDC was weighed out and added to give a final concentration of 10 mg/ml. The pH of the reaction mixture was adjusted and maintained at pH 5 with 1 N NaOH throughout the 5 minute incubation time at room temperature. An equal volume of an 11 mg/ml solution of protein carrier (BSA or porcine thyroglobulin) was added and the reaction was allowed to proceed at room temperature for 4 hours. The reaction was then stopped by the addition of a sodium acetate solution (1 M, pH 4.2) to give a final concentration of 100 mM. After an additional incubation of 1 hour at room temperature, the conjugate was dialyzed against 0.1 M phosphate buffer pH 7 for 24 hours using pre-hydrated SpectraPor cellulose ester membranes with molecular weight cut off of 15,000 (Spectrum Medical Industries Inc., Los Angeles, CA, USA). After dialysis, aliquots of the conjugates were stored at -20°C prior to lyophilization. Lyophilization of the conjugates was carried out at the Drug Delivery Laboratory, University of Florida, Progress Center, Alachua, FL, USA using a Model 12K Super Modulyo Lyophilizer (Edwards, West Sussex, England).

After lyophilization, samples of LE, BSA, porcine thyroglobulin and the conjugates were sent for amino acid analysis at the Peptide Core of the Interdisciplinary Center Biotechnology Research, University of Florida, Gainesville, FL, USA. The loading ratios of LE to carrier protein were calculated from the percentage composition of asparagine, threonine, serine and glutamine in the carriers and conjugates. The LE-thyroglobulin conjugate obtained by the glutaraldehyde method showed the highest peptide loading ratio (see Results and Discussion) and therefore this conjugate was selected as the inoculum for the purposes of this work.

For the initial inoculation, complete Freund's adjuvant was placed in a test tube and an equal volume of 0.1 M sodium phosphate buffer pH 7.4 containing 2 mg/ml of the LE-thyroglobulin conjugate was added while vortexing, to give a thick emulsion containing 1 mg/ml of the conjugate. Three young adult male New Zealand White rabbits each received a total of 1 ml of this emulsion subcutaneously at 6-8 different sites in the back. Thereafter, booster injections with the same dose of conjugate were given at monthly intervals in the same manner, except that incomplete Freund's adjuvant was used instead of complete Freund's adjuvant. Test bleeds were taken 7-10 days after each boost and after serum separation, the antibody titer was tested by radioimmunoassay. Briefly, to test total binding, [tyrosyl-3,5-^{3}H(N)]-leucine enkephalin (^{3}H-LE, 1 nM) was incubated overnight at 4°C with various dilutions of the antiserum (1/10, 1/100, 1/1,000, 1/10,000) in 0.1 M sodium phosphate buffer pH 7. To test non-specific binding, ^{3}H-LE (1 nM) was incubated overnight at 4°C with LE (500 nM) and various dilutions of the antiserum (1/10, 1/100, 1/1,000, 1/10,000) in 0.1 M sodium phosphate buffer pH 7. An ice-cold suspension containing 1.5 % w/v activated charcoal and 0.15 % w/v dextran in water was then added to each sample and after a 5 minute incubation at 4 °C, the samples were centrifuged at 12,000 g for 3 minutes. An aliquot of the supernatant was then removed, Cytoscint scintillation cocktail (4 ml) was added and the radioactivity (CPM) representing the antibody-bound tracer (^{3}H-LE) was determined using a Beckman LS 5,000 TD scintillation counter (Fullerton, CA, USA). After the third boost, the antibody titer of one of the rabbits was deemed sufficient and therefore, this rabbit was exsanguinated, the serum was separated from whole blood by centrifugation at 5,000g for 10 minutes (Dynac II Centrifuge, Clay Adams, Sparks, MD, USA) and stored in aliquots at -80°C.

A 10 ml aliquot of the anti-LE antiserum was purified by the Hybridoma Core of the Interdisciplinary Center Biotechnology Research, University of Florida, Gainesville, FL. Briefly, a column was filled with 10 ml of Prot G-Gammabind Ultra matrix (Pharmacia LKB Biotechnology Inc., Piscataway, NJ, USA) and washed with 35 ml of elution buffer (0.1 M glycine pH 3.0). The column was then washed extensively with 200-300 ml of binding buffer (0.1 M phosphate buffered saline pH 7, 0.01% sodium azide). The antiserum was diluted two-fold with binding buffer, loaded onto the column and washed with binding buffer until the optical density of the eluent reached zero at λ_{280}. Elution buffer was then added to the column, the eluent was collected in 1 ml fractions and neutralized immediately with 35 μl of 2 M Tris buffer pH 9. The fractions with high optical density at λ_{280} were pooled and then desalted and concentrated by spinning down 5-6 times for 30 minutes in a C-PREP 10 centrifugal filter (Amicon, Danvers, MA, USA). This procedure yielded 8.5 ml of purified anti-LE antibody containing 6.1 mg IgG/ml, determined by measurement of optical density at λ_{280}.

Characterization of Antibody

The number of specific antibody sites in the purified antibody was determined by means of Scatchard analysis [Scatchard 1949]. Samples containing various concentrations of ^{3}H-LE as tracer (70-1000 pM) and a 1/1,000 dilution of the purified antibody were set up to determine total binding (Table 2.1). To determine non-specific binding, samples containing antibody, ^{3}H-LE (70-1000 pM) and a high concentration of LE ($1.2*10^{-6}$ M) were also set up at the same time. After overnight incubation at 4°C, 200 μl of an ice cold suspension containing 1.5% activated charcoal and 0.15% dextran in water was added to each tube (except total counts) and after a further 5 minute incubation on ice, the samples were centrifuged at 12,000g for 3 minutes. Three hundred and fifty microliters of the resultant supernatant containing the antibody-bound fraction of the tracer were then removed and added to 4 ml of Cytoscint scintillation cocktail. The radioactivity (CPM) representing the antibody-bound tracer was determined using a Beckman LS 5,000 TD scintillation counter (Fullerton, CA, USA) and a 5 minute counting time with counting efficiency at about 50%.

Table 2.1. Samples set up for Scatchard analysis.

	Total counts	Total binding	Non-specific binding
Buffer	420 µl	20 µl	-----
^{3}H-LE	20 µl	20 µl	20 µl
LE (1.4*10^{-5} M)	-----	-----	20 µl
Antibody (1/1,000)	-----	200 µl	200 µl
Overnight at 4°C, then:			
Charcoal (1.5%)/dextran (0.15%)	-----	200 µl	200 µl

A Scatchard plot was constructed by plotting the ratio of bound to free ^{3}H-LE against bound ^{3}H-LE and the K_a value was determined from the negative slope of the line drawn between the points. The number of specific antibody sites was determined from the intercept of this line with the x-axis.

Biotinylation of Leucine Enkephalin

N-terminal biotinylated LE (BLE) was synthesized by allowing 90 nmoles of LE, 180 nmoles of N-hydroxysuccinimidobiotin (BHS) and 120 nmoles of triethanolamine in 150 µl of dimethylsulfoxide (DMSO) to incubate overnight at room temperature. The product of the reaction was separated from the reagents by injecting the incubation mixture into a gradient HPLC system consisting of a Rainin Rabbit HP solvent delivery system (Rainin Instrument Company Inc., Woburn, MA) controlled by a Rainin Dynamax HPLC method manager (version 1.3, Rainin Instrument Company Inc., Woburn, MA), a Negretti & Zamba injector (Southampton, UK) fitted with a 100 µl loop, a µBondapak C_{18} column (10 µm, 3.9 x 150 mm, Waters Associates, Milford, MA, USA) and a LDC

Milton Roy Spectromonitor 3100 variable wavelength detector (Riviera Beach, FL, USA). The detection wavelength was set at 210 nm. Mobile phase A consisted of 90 % v/v aqueous trifluoroacetic acid (0.02% v/v) and 10 % v/v acetonitrile and mobile phase B consisted of 10 % v/v aqueous trifluoroacetic acid (0.02% v/v) and 90 % v/v acetonitrile. A gradient of 90 % mobile phase A and 10 % mobile phase B to 50 % each of mobile phases A and B in 30 minutes was run at a flow rate of 1 ml/min. A control injection of the incubation mixture was also made into the HPLC system immediately after preparation to allow the calculation of the extent of conversion of LE to BLE. N-terminal biotinylated LE incorporating a spacer arm between the biotin group and the peptide (BXLE) was synthesized in the same manner except that biotinamidocaproate N-hydroxysuccinimide ester (BXHS) was used instead of BHS in the incubation mixture. The peaks corresponding to BLE and BXLE were collected from the HPLC eluent and the volatile components (acetonitrile and trifluoroacetic acid) were evaporated under a stream of nitrogen at 30°C. The biotinylated LE derivatives were stored at 4°C and were used within two weeks of synthesis.

C-terminal biotinylated leucine enkephalin-Lys^6 (LE-Lys^6-B) was synthesized by allowing 73 nmoles of leucine enkephalin-Lys^6 (LE-Lys^6), 142 nmoles of BHS and 90 nmoles of triethanolamine in 150 μl of DMSO to incubate for two hours at room temperature. The product of the reaction was separated from the reagents by injecting this incubation mixture into the same gradient HPLC system described above except that a gradient of 90 % mobile phase A and 10 % mobile phase B to 70 % mobile phase A and 30% mobile phase B in 30 minutes was run at a flow rate of 1 ml/min. A control injection of 73 nmoles of LE-Lys^6 in 150 μl of DMSO was also made into the HPLC system to allow the calculation of the extent of conversion of LE-Lys^6 to LE-Lys^6-B. C-terminal biotinylated LE incorporating a spacer arm between the biotin group and the peptide (LE-Lys^6-BX) was synthesized in the same manner except that BXHS was used instead of BHS in the incubation mixture. The peaks corresponding to LE-Lys^6-B and LE-Lys^6-BX were collected from the HPLC eluent and stored as described earlier for BLE and BXLE.

The successful biotinylation of LE by the methods described above was characterized by comparing the displacement of 2-(4'-hydroxyazobenzene)-benzoic acid (HABA) from avidin by the biotinylated LE derivatives and biotin itself according to the spectrophotometric method of Green [Green 1970]. A 0.25 mM solution of HABA was prepared in 0.1 M sodium phosphate buffer pH 7 and avidin was added to an aliquot of this solution to give a final concentration of 45 μM. One milliliter of this solution was placed in a UV quartz cuvette and upon serial additions of the biotinylated species or biotin as a positive control, the absorbance at λ_{500nm} was monitored using a Cary 3E UV-Visible spectrophotometer (Varian, Sugarland, TX, USA). The same experiment was repeated using each biotinylated LE derivative.

The structures of LE-Lys^6-B and LE-Lys^6-BX were confirmed by mass spectrometric analysis by the Protein Chemistry Core of the Interdisciplinary Center for Biotechnology Research, University of Florida, Gainesville, FL, USA. A Lasertech matrix-assisted laser desorption (MALDI) - time-of-flight (TOF) instrument operated at 10 kV acceleration was used with a 335 nm UV laser and α-cyano-4-hydroxycinnamic acid as the matrix. The sample was dissolved in a solution (acetonitrile/water, 1/2 v/v) containing a large excess of the matrix (5g/l) and a 1 µl aliquot was deposited onto the stainless steel laser target. Several spectra were averaged for each experiment.

Antibody-Binding of LE, Biotinylated LE Derivatives and Preformed Biotinylated LE-Avidin Complexes

The binding of LE, the biotinylated LE derivatives and preformed biotinylated LE-avidin complexes to both a commercial anti-LE antiserum (Peninsula) and the antibody produced in this laboratory was tested using a standard radioimmunoassay procedure. For the assays using preformed biotinylated LE-avidin complexes, a ten-fold molar excess of avidin was added to the highest concentration of biotinylated LE in buffer and vortexed immediately. The complexes were allowed to form for 30 minutes prior to serial dilution to give the various concentrations of competitor required for the assay. The assays were set up in microcentrifuge tubes as shown in Table 2.2. The RIA buffer used consisted of 0.1 M sodium phosphate buffer pH 7 containing 0.1% w/v BSA. The tracer was ^{3}H-LE ($2.6*10^{-10}$ M final concentration) and the competitor was various concentrations of either LE, the biotinylated LE derivatives or preformed biotinylated LE-avidin complexes. After overnight incubation at 4°C, 200 µl of an ice cold suspension containing 1.5% w/v activated charcoal and 0.15% w/v dextran in water was added to each tube (except total counts) and after a further 5 minute incubation on ice, the samples were centrifuged at 12,000g for 3 minutes. Three hundred and fifty microliters of the resultant supernatant containing the antibody-bound fraction of the tracer was then removed and added to 4 ml of Cytoscint scintillation cocktail. The radioactivity (CPM) representing the antibody-bound tracer was determined using a Beckman LS 5,000 TD scintillation counter (Fullerton, CA, USA).

Table 2.2. Radioimmunoassay set up

	Total counts	Total binding	Sample
RIA Buffer	420 µl	20 µl	-----
Tracer ($3.125*10^{-9}$ M)	20 µl	20 µl	20 µl
Competitor	-----	-----	20 µl
Antibody dilution	-----	200 µl	200 µl
Overnight at 4°C, then:			
Charcoal (1.5%)/dextran (0.15%)	-----	200 µl	200 µl

The IC_{50} values (concentration of competitor displacing 50% of bound tracer) for LE, the biotinylated LE derivatives or preformed biotinylated LE-avidin complexes were determined using the MINSQ non-linear curve-fitting program (MicroMath Scientific Software, Salt Lake City, UT, USA). The data were fitted to the following model:

$$B = T - \frac{T * C^N}{C^N + IC_{50}^N} + NS$$

Where: B = CPM in the presence of competitor
T = CPM in the absence of competitor
C = competitor concentration
N = slope factor
NS = CPM under non-specific binding conditions

As far as possible, non-specific binding was determined in the presence of relatively high concentrations of competitor (2-3 orders of magnitude greater than the

IC_{50} value). When high enough concentrations of competitor could not be achieved to determine non-specific binding, the non-specific binding value obtained from the LE curve was used to fit the curve for the other competitors.

Development of the Proposed Homogeneous Fluorescence Immunoassay

Determination of excitation and emission maxima for fluorescence-labeled avidin

Fluorescein isothiocyanate avidin (FITC-avidin) was used as the "detector" molecule in this assay. To determine the excitation and emission maxima, 2 ml of a solution of FITC-avidin (7.5 pmol/ml) were dispensed into a quartz fluorescence cuvette and scans of the excitation and emission spectra were carried out using a Perkin Elmer LS-3B fluorescence spectrophotometer (Norwalk, CT, USA). The scans were then repeated upon addition of BLE (70 pmol/ml) to determine the fluorescence enhancement produced on interaction of these two reagents.

Determination of reagent concentrations

The results of the binding experiments described above indicated that the antibody made in this laboratory in combination with the N-terminal biotinylated derivative without the spacer arm (BLE) should be used in the development of the proposed homogeneous fluorescence immunoassay for LE (see Results and Discussion). One of the first steps in the development of this assay lay in the determination of the concentrations of antibody, BLE and FITC-avidin to be used.

The decision as to the concentration of antibody to be used in the final assay ($3.78*10^{-9}$ M, 1/50 dilution of purified antibody) was made based on practicality since it was anticipated that a large amount of antibody would be required for the development of the homogeneous fluorescence immunoassay and a limited quantity of antibody, obtained through the exsanguination of a single rabbit was available.

To ensure low background fluorescence readings, the amount of BLE used in the final assay needed to be such that close to 100 % could be bound by the antibody present. When BLE and antibody (Ab) are present in the same solution, the equilibrium established can be described as:

$$\text{BLE} + \text{Ab} \rightleftharpoons \text{BLE-Ab}$$

According to the Law of Mass Action, the following equation can be set up:

$$K_a = \frac{[BLE - Ab]}{[BLE][Ab]}$$

Which can be rearranged to give:

$$\frac{[BLE]}{[BLE - Ab]} = \frac{1}{[Ab]K_a}$$

If 95% of the BLE in the assay is to be bound by the amount of antibody chosen for the assay ($3.78 * 10^{-9}$ M, 1/50 dilution of purified antibody), the left side of the above equation can be set to equal $\frac{5}{95}$. Since [Ab] = [Ab_{total}] - [BLE-Ab], by using the K_a value obtained from the Scatchard plot described earlier (assuming that BLE and LE have the same affinity for the antibody), the above equation can be solved for [BLE-Ab]. [BLE-Ab] represents 95% of the concentration of BLE to be used in the assay and therefore, the total amount of BLE to be used in the final assay for 95% to be bound by the chosen antibody concentration could be calculated.

The concentration of FITC-avidin to be used in the assay was determined by finding the concentration of this reagent which will give maximum fluorescence enhancement with the chosen concentration of BLE. Various concentrations of FITC-avidin (200-1000 fmol/ml) in 0.1 M sodium phosphate buffer pH 7 were allowed to interact with various concentrations of BLE (0.1-20 pmol/ml) for 20 minutes, after which time, fluorescence readings were taken at λ_{exc} 482 nm and λ_{em} 517 nm using a Perkin Elmer LS-3B fluorescence spectrophotometer (Norwalk, CT, USA).

Homogeneous fluorescence immunoassay

Once the concentrations of the reagents to be used were determined, the complete homogeneous fluorescence immunoassay samples were set up as shown in Table 2.3. Total tracer samples (4 pmol BLE per ml of buffer) were also prepared to give an indication of the maximum fluorescence enhancement which could be expected due to the interaction of the total amount of tracer present with FITC-avidin. Fluorescence blank samples were set up to determine the fluorescence due to the native fluorescence of FITC-avidin present in each sample. The buffer used consisted of 0.1 M sodium phosphate buffer pH 7 containing 0.1% w/v BSA. After overnight incubation at 4°C, 20 µl of FITC-avidin (13 pmol/ml) were added to each sample to give a final concentration of 500 fmol/ml. The interaction between the free tracer (BLE) and the "detector" molecule

Table 2.3. Homogeneous fluorescence immunoassay set-up.

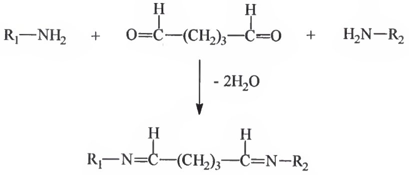
	Total tracer	Total binding	Sample	Fl blank
Buffer	450 µl	350 µl	various	500 µl
BLE (40 pmol/ml)	50 µl	50 µl	50 µl	----
LE	----	----	various	----
Purified antibody (1/10)	----	100 µl	100 µl	----
Total volume	500 µl	500 µl	500 µl	500 µl

(FITC-avidin) was allowed to proceed for 20 minutes at room temperature in the dark, after which fluorescence readings were taken at λ_{exc} 482 nm and λ_{em} 517 nm using a Perkin Elmer LS-3B fluorescence spectrophotometer (Norwalk, CT, USA).

Results and Discussion

Antibody Production

It was anticipated that the development of the homogenous fluorescence immunoassay in particular would require large quantities of antibody. Therefore, it was decided to produce a polyclonal antibody to leucine enkephalin in this laboratory.



$$R_1-NH_2 + O=\overset{H}{C}-(CH_2)_3-\overset{H}{C}=O + H_2N-R_2 \xrightarrow{-2H_2O} R_1-N=\overset{H}{C}-(CH_2)_3-\overset{H}{C}=N-R_2$$

Figure 2.3. Coupling of peptide to protein carrier using glutaraldehyde. R_1= peptide or protein carrier, R_2= peptide or protein carrier.

As LE is not antigenic itself, the peptide had to be conjugated to a protein carrier in order to stimulate an immune reaction in the host animal. Glutaraldehyde is a bifunctional coupling reagent that binds two compounds primarily through their amino groups. Since LE contains only one amino group at its N-terminal end, using the glutaraldehyde method of conjugation, the peptide is conjugated to the protein carrier via the N-terminal end (Figure 2.3). Therefore, on inoculation with such a conjugate, one would expect the antibody that is produced to be directed against the C-terminal end of the peptide.

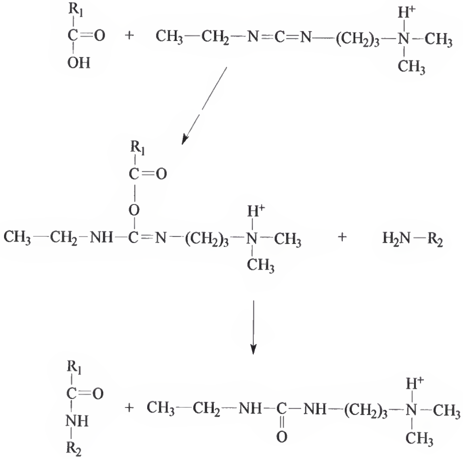


Figure 2.4. Coupling of peptide to protein carrier using EDC. R_1=peptide or protein carrier, R_2=peptide or protein carrier.

In contrast, EDC allows the conjugation of the peptide to the protein carrier via either the N-terminal or the C-terminal end as carbodiimides attack carboxylic groups to change them into reactive sites for free amino groups. Therefore, the carboxylic groups on the protein carrier will conjugate to LE via the N-terminal amino group of the peptide and LE will also conjugate to the protein carrier through its C-terminal end by attacking the free amino groups on the protein carrier (Figure 2.4). As a result, on inoculation with a conjugate produced by the EDC method, one could expect antibodies directed against either or both the N-terminal or the C-terminal end of LE.

Table 2.4. Loading ratios of LE to protein carrier by different conjugation methods.

Protein carrier	Conjugation method	LE : protein ratio
Porcine thyroglobulin	Glutaraldehyde	545 : 1
Porcine thyroglobulin	EDC	70 : 1
Bovine serum albumin	Glutaraldehyde	60 : 1
Bovine serum albumin	EDC	1.37 : 1

The loading ratios of peptide to protein carrier calculated from the results of amino acid analysis are shown in Table 2.4. Due to the high immunogenicity of porcine thyroglobulin in rabbits and the fact that the LE-thyroglobulin conjugate obtained by the glutaraldehyde method showed the highest peptide loading ratio, this conjugate was selected as the inoculum for the purposes of this work.

The results of the radioimmunoassay carried out after the third boost showed that when a 1/1,000 dilution of the antiserum was used, 30% of the total radioactivity added was bound and 32% of this bound radioactivity was determined to be due to non-specific binding (Figure 2.5). At that time, this titer was deemed to be sufficient and therefore the antiserum was harvested.

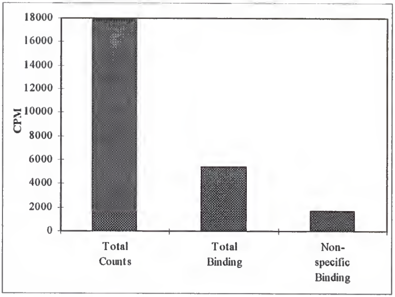


Figure 2.5. Bar graph showing total counts, total binding and non-specific binding of ^{3}HLE using 1/1,000 dilution of antibody produced in this laboratory.

Characterization of Antibody

The Scatchard plot of bound/free ^{3}H-LE versus bound ^{3}H-LE is shown in Figure 2.6. The K_a value determined from the negative slope of the line drawn between the points was $3.87*10^9$ l/mol which converts to a K_d value of $2.58*10^{-10}$ mol/l. The number of specific antibody sites present in the final incubation for this experiment was determined from the x-axis intercept to be $3.62*10^{-10}$ mol/l. Since $3.18*10^{-8}$ mol/l of IgG was used in this preparation, the purified antibody contains only 1.14% specific antibody sites. By extrapolation, the stock solution of purified antibody was determined to contain $4.35*10^{-7}$ mol/l of specific antibody sites. The affinity of the antibody made in this laboratory to LE compared favorably to the commercial antiserum obtained from Peninsula under the same assay conditions since IC_{50} values for LE using a 1/1,000 dilution of the antibody produced in this laboratory ($3.8*10^{-13}$ mol/assay) was similar to those obtained using a 1/3,000 dilution of the commercial antiserum ($1.3*10^{-13}$ mol/assay). Peninsula claimed that IC_{50} values of $1.4*10^{-14}$ mol/assay could be achieved using a 1/15,000 dilution of their antiserum. However, their assay conditions involved the use of a tracer labeled with ^{125}I instead of ^3H. ^{125}I is a higher energy label than ^3H, and therefore, Peninsula was able to use a lower concentration of tracer in their assay, which allowed them to use less antiserum, resulting in a lower IC_{50} value.

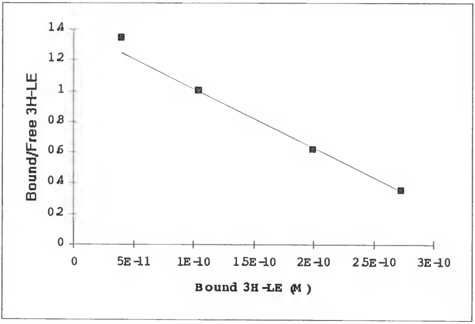


Figure 2.6. Scatchard plot of bound/free 3H-LE versus bound 3H-LE

Biotinylation of Leucine Enkephalin

Since one of the proposed immunoassays required the formation of a sandwich between the anti-LE antibody, the biotinylated LE derivative and fluorescence- or enzyme-labeled avidin, and the other did not, several different biotinylated derivatives were synthesized and tested. Some of the biotinylated derivatives included a spacer arm between the biotin group and the peptide (Figure 2.8) as it was felt that this would increase the likelihood of the formation of a sandwich between the anti-LE antibody, the biotinylated LE derivative and enzyme-labeled avidin by reducing steric hindrance. Biotinylation took place at the N-terminal end of the peptide when LE was used as a starting material as the only free amino group available for the reaction is the N-terminal amino group of the peptide. When LE-Lys^6 was used as a starting material, biotinylation took place primarily at the C-terminal end of the peptide as here, the amino group of the lysine moiety is more reactive than the N-terminal amino group under the reaction conditions used due to its greater basicity.

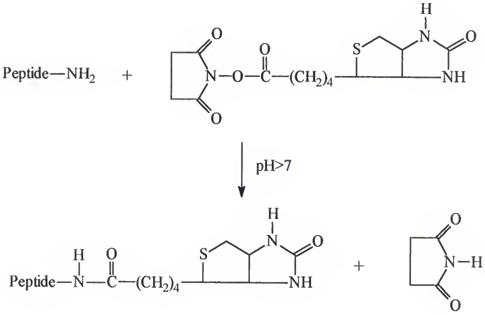


Figure 2.7. Biotinylation of peptide using N-hydroxysuccinimidobiotin.

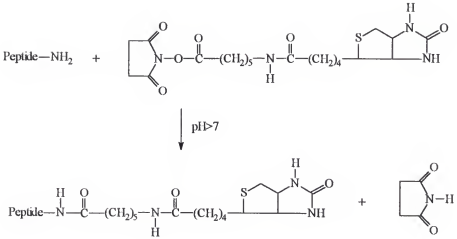


Figure 2.8. Biotinylation of peptide using biotinamidocaproate N-hydroxysuccinimide ester.

The reactants and products of the biotinylation reactions were successfully separated from each other using the HPLC systems described previously (Figures 2.9 and 2.10). In control injections containing LE or LE-Lys^6 in concentrations equal to the initial concentrations of these compounds in the reaction mixture, the reactants were seen to elute after 9 minutes (Figure 2.9A) and 5.15 minutes (Figure 2.10A), respectively. The N-terminal biotinylated LE derivatives, BLE (no spacer arm) and BXLE (with spacer arm) were seen to elute distinct from the reactants at 15.3 and 16.3 minutes, respectively (Figures 2.9B and 2.9C). For the C-terminal biotinylation of LE-Lys^6 to give LE-Lys^6-B (no spacer arm) and LE-Lys^6-BX (with spacer arm), two product peaks were seen on injection of the incubation mixtures. Based on the delayed formation in the time course of the reaction of the minor peak eluting at 24.8 minutes in Figure 2.10B and at 27.8 minutes in Figure 2.10C, these minor peaks were assumed to be di-biotinylated products biotinylated at both the N- and C-terminal ends of the peptide. Analysis by mass spectro-

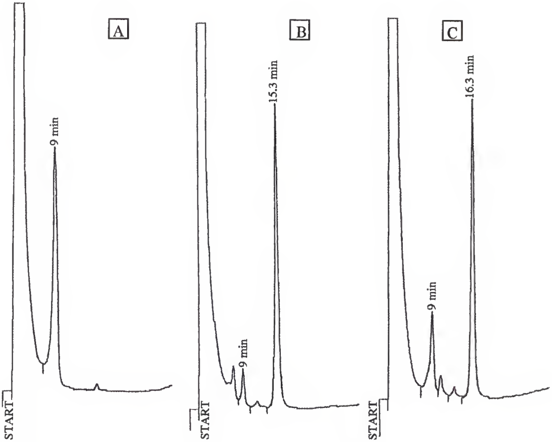


Figure 2.9. Chromatographs of control injection (A) showing LE eluting after 9 minutes and reaction injections showing BLE (no spacer arm) eluting after 15.3 minutes (B) and BXLE (with spacer arm) eluting after 16.3 minutes (C).

metry (MALDI) of the major peak eluting at 14.9 minutes in Figure 2.10B and at 17.8 minutes in Figure 2.10C revealed molecular ion peaks ($[M+H]^+$) at m/z 910.283 and 1022.1, respectively. The nominal molecular masses of the molecular ions of LE-Lys^6-B and LE-Lys^6-BX were calculated to be 910.11 and 1023.27, respectively. Since the mass determinations deviated from the nominal masses of mono-biotinylated derivatives by less than 0.1%, these products were confirmed as mono-biotinylated derivatives of LE-Lys^6. Therefore, antibody binding experiments were carried out using these collected peaks. The

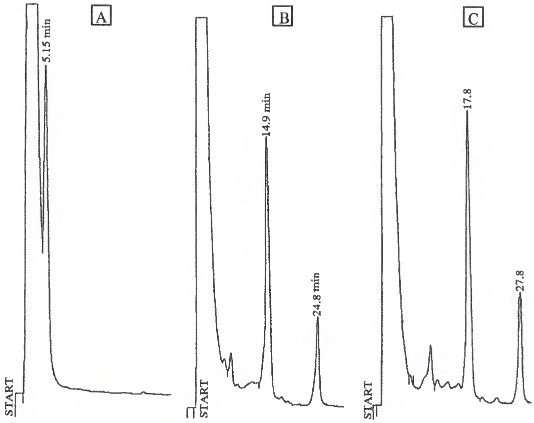


Figure 2.10. Chromatographs of control injection (A) showing LE-Lys^6 eluting after 5.15 minutes and reaction injections showing LE-Lys^6-B (no spacer arm) eluting after 14.9 minutes (B) and LE-Lys^6-BX (with spacer arm) eluting after 17.8 minutes (C). Di-biotinylated products are seen eluting at 24.8 minutes (B) and 27.8 minutes (C).

extent of conversion from the starting materials (LE and LE-Lys^6) to the biotinylated derivatives was estimated from the difference in peak height of LE and LE-Lys^6 in the control injection and in the injection of the reaction mixture. An extent of conversion of approximately 80% from the starting materials to the biotinylated derivatives was estimated. The results of the HABA displacement experiments confirmed that the products collected from the HPLC eluent were indeed biotinylated LE derivatives as they were seen to displace HABA from avidin (Figure 2.11). The results of this experiment confirm that

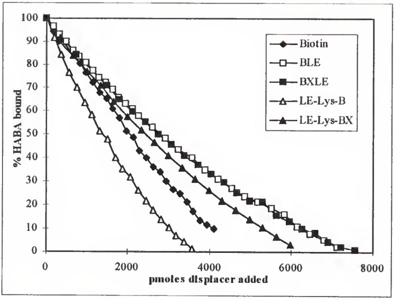


Figure 2.11. Displacement of HABA from avidin by biotin and biotinylated LE derivatives.

on conjugation to LE, biotin retains the ability to bind to avidin. The curvilinear nature of the displacement curves for BLE, BXLE and LE-Lys^6-BX together with the observation that more of these biotinylated derivatives compared to biotin is required to displace the same amount of HABA from avidin indicates that these derivatives have a slightly lower affinity for avidin than biotin itself. However, here, LE-Lys^6-B is seems to have a slightly higher affinity to avidin than biotin. This effect can probably be attributed to experimental error as the concentrations of the biotinylated derivatives were calculated from estimated conversion rates based on the reduction of the peak heights of the starting materials in the biotinylation reaction. Previous investigations on the binding of biotinylated peptides or other macromolecules to avidin have shown that a spacer arm may be required to retain full affinity to avidin [Finn et al. 1984, Green et al. 1971], however this observation was not reflected in the results seen here as LE biotinylated with and without the spacer arm both showed similar affinity to avidin and in fact, LE-Lys^6 biotinylated without the spacer arm showed a higher affinity to avidin than LE-Lys^6 biotinylated with the spacer arm (Figure 2.11). This may be attributed to the fact that LE and LE-Lys^6 are rather small and therefore, on conjugation to biotin, they do not cause steric hindrance to the binding of biotin to avidin.

Antibody-Binding of LE, Biotinylated LE Derivatives and Preformed Biotinylated LE-Avidin Complexes

Using a 1/3,000 dilution of the commercial antiserum, binding curves were constructed for LE, the N-terminal biotinylated derivatives, the C-terminal biotinylated derivatives and the corresponding pre-formed complexes with avidin. The IC_{50} values obtained for LE, BLE, BXLE, BLE-avidin and BXLE-avidin are shown in Table 2.5. Representative binding curves for LE, BLE, BXLE, BLE-avidin and BXLE-avidin are shown in Figure 2.12.

Table 2.5. IC_{50} values obtained for LE, BLE, BXLE, BLE-avidin and BXLE-avidin using 1/3,000 dilution of commercial antiserum.

Competitor	IC_{50} (mol/assay)	Mean	Standard deviation
LE	$1.15*10^{-13}$ $1.04*10^{-13}$ $1.58*10^{-13}$	$1.3*10^{-13}$	$2.7*10^{-14}$
BLE	$4.44*10^{-14}$ $3.56*10^{-13}$	$2.0*10^{-13}$	-----
BXLE	$4.42*10^{-15}$ $2.26*10^{-14}$	$1.3*10^{-14}$	-----
BLE-avidin	$1.89*10^{-10}$	-----	-----
BXLE-avidin	$1.46*10^{-11}$	-----	-----

The C-terminal biotinylated LE derivatives LE-Lys^6-B and LE-Lys^6-BX showed no binding to the commercial antiserum in the concentrations used (data not shown) and therefore, using this preparation, they were unsuitable for the development of either of the immunoassays proposed in this study. It is possible that the inclusion of a longer spacer arm in the C-terminal biotinylated derivatives would have allowed binding of the commercial antiserum if the epitope to be recognized by the antibody remained intact on biotinylation. However, this avenue was not explored.

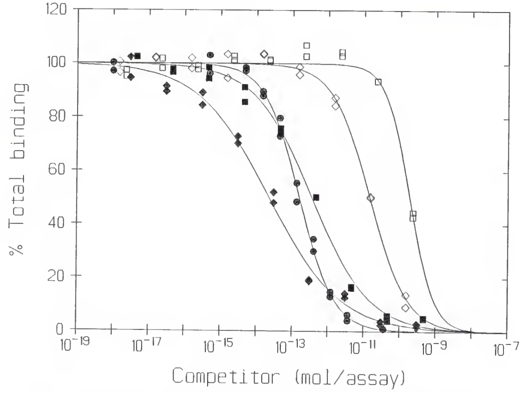


Figure 2.12. Representatives binding curves for LE (●), BLE (■), BXLE (◆), BLE-avidin (□) and BXLE-avidin (◊) using 1/3,000 dilution of commercial antiserum.

In these experiments, BXLE showed a ten-fold higher affinity to the commercial antiserum than LE (Table 2.5). It was postulated that since LE needs to be conjugated to a protein carrier to render it immunogenic for antibody production, this biotinylated LE derivative including a spacer arm may resemble the epitope presented to the immune system more closely than LE itself and therefore, BXLE shows a higher affinity to the commercial antiserum than LE itself.

Although the N-terminal biotinylated derivatives were seen to retain affinity for the commercial antiserum, a shift in affinity by 2 or 3 orders of magnitude was seen when complexes were formed between BLE or BXLE and avidin (Table 2.5), indicating that a sandwich was not formed between the commercial antiserum, BLE or BXLE and avidin. Therefore, this commercial antiserum was deemed to be unsuitable for the development of the proposed ELISA for which sandwich formation is a requirement. Although this lack of sandwich formation indicates that this preparation is suitable for the development of the proposed homogeneous fluorescence immunoassay, as it was anticipated that large quantities of antibody would be required for the development of this assay, this avenue was unfeasible. As a result, efforts involving the development of either an ELISA or an homogeneous fluorescence immunoassay using the commercial antiserum preparation were abandoned.

Using a 1/1,000 dilution of the antibody produced in this laboratory, binding curves were also constructed for LE, the N-terminal biotinylated LE derivatives, the C-terminal biotinylated LE derivatives and the corresponding pre-formed complexes with avidin. The IC_{50} values obtained for LE, BLE, BXLE and BXLE-avidin are shown in Table 2.6. Representative binding curves for LE, BLE, BXLE and BXLE-avidin are shown in Figure 2.13.

Table 2.6. IC_{50} values obtained for LE, BLE, BLE-avidin, BXLE and BXLE-avidin using 1/1,000 dilution of antibody produced in this laboratory. [†]IC50 value for BLE-avidin was estimated by fixing non-specific binding to 0% total binding and N to 1.

Competitor	IC_{50} (mol/assay)	Mean	Standard deviation
LE	$4.95*10^{-13}$ $3.70*10^{-13}$ $3.78*10^{-13}$ $3.44*10^{-13}$ $2.79*10^{-13}$ $4.19*10^{-13}$	$3.8*10^{-13}$	$7.3*10^{-14}$
BLE	$6.22*10^{-13}$ $1.21*10^{-12}$ $1.38*10^{-12}$	$1.1*10^{-12}$	$4.0*10^{-13}$
BLE-avidin	[†]$3.73*10^{-11}$	-----	-----
BXLE	$3.53*10^{-14}$ $4.97*10^{-15}$	$2.0*10^{-14}$	-----
BXLE-avidin	$1.70*10^{-11}$ $1.11*10^{-11}$	$1.4*10^{-11}$	-----

As with the commercial antiserum, the N-terminal biotinylated derivatives were seen to retain affinity for the antibody produced in this laboratory. However, a shift in affinity by almost three orders of magnitude is seen when complexes are formed between BXLE and avidin (Table 2.6), and pre-formed BLE-avidin complexes showed binding to this antibody at only the highest concentration tested (Figure 2.13).

Again, as with the commercial antiserum, BXLE showed a higher affinity than LE to the antibody produced in this laboratory. Here too, as before, it was postulated that since LE was conjugated to a porcine thyroglobulin to render it immunogenic for antibody production, this biotinylated LE derivative including a spacer arm may have resembled the epitope presented to the immune system more closely than LE itself and as a result, BXLE also showed a higher affinity to this antibody than LE itself.

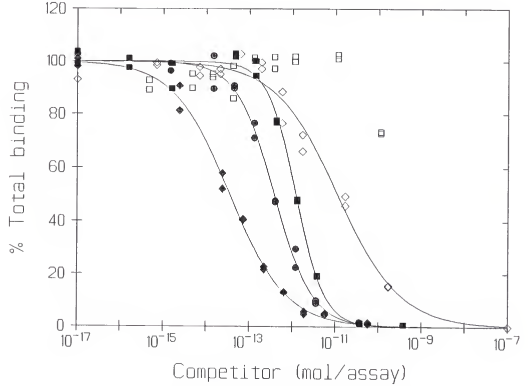


Figure 2.13. Representatives binding curves for LE (●), BLE (■), BXLE (◆), BLE-avidin (□) and BXLE-avidin (◊) using 1/1,000 dilution of antibody produced in this laboratory.

The C-terminal biotinylated LE derivatives LE-Lys^6-B and LE-Lys^6-BX showed no binding to the antibody produced in this laboratory in the concentrations used (data not shown) and therefore, in this preparation, they were also unsuitable for the development of either of the immunoassays proposed in this study. This result was expected since the antibody was produced in this laboratory following inoculation with LE conjugated to a carrier protein via the N-terminal end. Therefore, the C-terminal end of the peptide was exposed as an epitope to produce an immune response in the host animal, resulting in the production of antibodies towards this end of the peptide. Since LE-Lys^6-B and LE-Lys^6-BX are modified at the C-terminal end, one would naturally not expect them to bind to an antibody directed towards the C-terminal end of LE.

The formation of a sandwich between antibody, biotinylated LE derivative and avidin was not achieved using either the antibody produced in this laboratory or the commercial antiserum and any of the biotinylated LE derivatives synthesized. Since sandwich formation is a requirement for the successful development of the ELISA proposed in this study, efforts in this direction were abandoned at this point.

A lack of sandwich formation was observed between the antibody produced in this laboratory, BLE and avidin, and therefore, it was concluded that this combination of reagents was suitable for the development of the homogeneous fluorescence immunoassay proposed in this study.

Development of the Proposed Homogeneous Fluorescence Immunoassay

Determination of excitation and emission maxima for fluorescence-labeled avidin

The excitation maximum for FITC-avidin was determined to be 482 nm. Figure 2.14 below shows the emission scans for FITC-avidin and FITC-avidin with BLE. Maximum emission was observed at 517 nm and fluorescence was seen to increase by a factor of 4 when FITC-avidin interacted with BLE. Therefore, λ_{exc} 482 nm and λ_{em} 517 nm were chosen for future fluorescence readings.

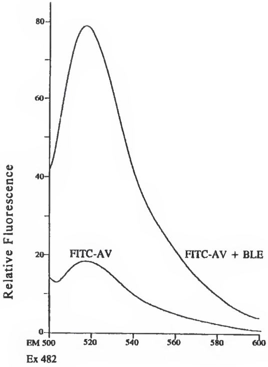


Figure 2.14. Emission scans with λ_{exc} at 482 nm for FITC-avidin and FITC-avidin with BLE.

Determination of reagent concentrations

An antibody dilution of 1/50 corresponding to a concentration of 8.7 pmol/ml of specific antibody sites was chosen for use in these experiments. Although a higher concentration of specific antibody sites in the final assay would have been preferred to afford binding of a greater number of tracer (BLE) molecules, the decision was based on practicality since a limited amount of antibody was available from the exsanguination of a single rabbit.

The calculations using the chosen antibody concentration, the K_a value obtained from the Scatchard plot and the Law of Mass Action indicated that a concentration of

BLE of 4 pmol/ml would be 95 % bound by the chosen antibody concentration. Therefore, this concentration of tracer was chosen for use in all further experiments.

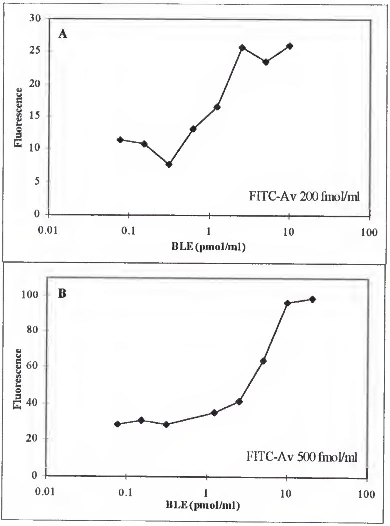


Figure 2.15. Plots of fluorescence versus amount of BLE added using 200 fmol/ml of FITC-avidin (A) and 500 fmol/ml of FITC-avidin (B)

Although one would expect that about 4 times as much BLE as FITC-avidin would produce maximum fluorescence enhancement of FITC-avidin since it is known that 4 moles of biotin will bind to 1 mole of avidin, Figure 2.15A shows that a maximum fluorescence reading is obtained when 4 pmol/ml of BLE is added to 200 fmol/ml of

FITC-avidin. The results of the HABA displacement experiments (Figure 2.11) indicated that BLE does have a lower affinity than biotin for avidin and in addition, since here FITC-avidin is being used, one might expect BLE to exhibit a lower affinity for FITC-avidin than biotin exhibits for avidin. In the plot shown in Figure 2.14A, the working scale is rather narrow and the curve obtained is not smooth as considerable noise interfered with the taking of the readings. Therefore, although a maximum fluorescence reading using 500 fmol/ml of FITC-avidin is seen at 10 pmol/ml rather than 4 pmol/ml of BLE (Figure 2.15B), this concentration of FITC-avidin was chosen for use in this assay since less noise is observed in the readings and the working scale is more practical.

Homogeneous fluorescence immunoassay

One of the first calibration curves obtained for LE using the complete homogeneous fluorescence immunoassay is shown in Figure 2.16. Here, as expected, an increase in fluorescence is seen with an increase of LE present in the sample. However, using the LE concentrations shown here, a plateau at the maximum theoretical fluorescence (total tracer value, no antibody present) was not reached. Therefore, another calibration curve was constructed using higher concentrations of LE in the samples in an attempt to reach this plateau (Figure 2.16).

Figure 2.17 shows a calibration curve for LE using the complete homogeneous fluorescence immunoassay where fluorescence readings above the maximum theoretical fluorescence were obtained at higher concentrations of LE ($1*10^{-8}$ moles/ml and above). In order to determine whether this unexpected effect could be attributed to either the antibody, the tracer or the analyte, the assay was repeated omitting each of these reagents in turn.

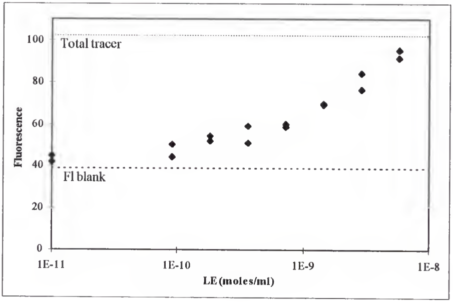


Figure 2.16. Calibration curve for LE using homogeneous fluorescence immunoassay.

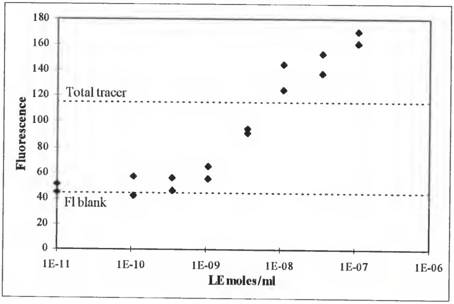


Figure 2.17. Calibration curve for LE using homogeneous fluorescence immunoassay showing readings exceeding maximum theoretical fluorescence.

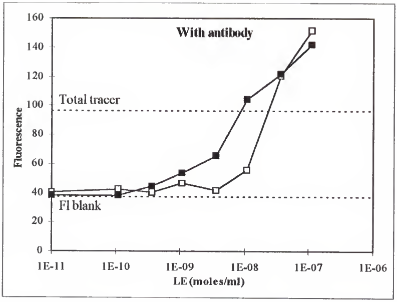


Figure 2.18. Calibration curve for LE using the homogeneous fluorescence immunoassay set up with BLE (■) and without BLE(□).

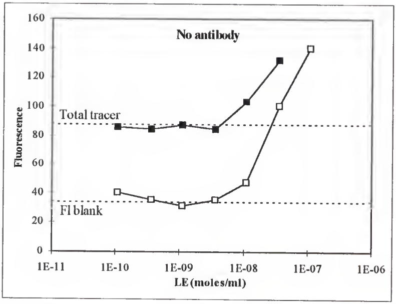


Figure 2.19. Calibration curve for LE using the homogeneous fluorescence immunoassay set up, omitting antibody but with BLE (■) and without BLE(□).

Figure 2.18 shows that at in the presence of the antibody, when the tracer (BLE) is omitted from the assay, fluorescence readings began to increase above the fluorescence blank levels at concentrations of LE in excess of $1*10^{-8}$ moles/ml. This effect was also observed when the antibody was omitted from the assay (Figure 2.19). When both tracer and antibody were included in the assay (Figure 2.18), fluorescence levels began to increase above fluorescence blank levels at concentrations of LE in excess of $1*10^{-9}$ moles /ml. When antibody is omitted but tracer is included in the assay, fluorescence readings increased above total tracer levels at LE concentrations in excess of $1*10^{-8}$ moles/ml (Figure 2.19). This is the same concentration of LE at which the fluorescence readings increased above fluorescence blank levels when both tracer and antibody were omitted from the assay.

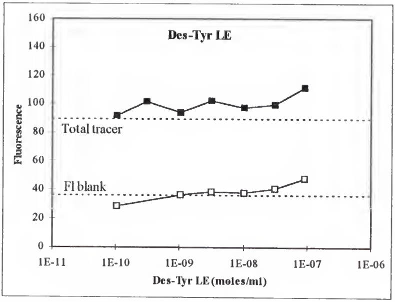


Figure 2.20. Calibration curve for des-Tyr[1] LE using the homogeneous fluorescence immunoassay set up, omitting antibody but with BLE (■) and without BLE(□).

These results led us to believe that at higher concentrations of LE, the analyte interacted directly with FITC-avidin to give increased fluorescence readings. It was hypothesized that this effect could be due to an interaction between the tyrosine moiety in the 1 position of LE and the fluorescein groups of the "detector" molecule FITC-avidin. In order to test this hypothesis, assays were carried out in the absence of antibody, in the same way as described above. However, here des-Tyr[1] LE or pentaglycine were used as analytes in concentrations similar to those of LE which had been used previously.

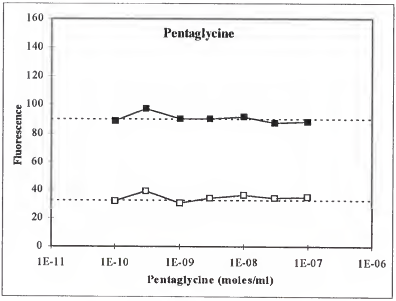


Figure 2.21. Calibration curve for pentaglycine using the homogeneous fluorescence immunoassay set up, omitting antibody but with BLE (■) and without BLE(□).

Figures 2.20 and 2.21 show that des-Tyr[1] LE and pentaglycine do not produce the same interaction with FITC-avidin as LE when used in the same concentrations in the homogeneous immunoassay set up. When BLE is included in the assay, the readings approach those obtained in the total tracer samples and when no BLE is present in the assay, the readings approach those in the fluorescence blank samples. It was therefore concluded that the effect seen at higher concentrations of LE in the homogenous fluorescence immunoassay were probably due to an interaction between the tyrosine moiety of LE and the fluorescein groups of FITC-avidin.

The homogenous fluorescence immunoassay evaluated here operates as intended in the concentration range between $1*10^{-9}$ moles/ml and $1*10^{-8}$ moles/ml of LE since at these concentrations, sufficient tracer (BLE) is displaced from the antibody by LE to produce detectable fluorescence enhancement on interaction with the detector molecule (FITC-avidin), but the concentration of LE is not high enough to produce a direct interaction with FITC-avidin in the absence of BLE. Using the reagents tested, an homogeneous fluorescence immunoassay for LE operational over a wide concentration range could not be developed since at higher concentrations ($1*10^{-8}$ moles/ml and above), LE interacted directly with FITC-avidin to produce fluorescence enhancement, thus interfering with the signal produced through the interaction of BLE with FITC-avidin. In this assay, the use of an antibody with a higher affinity for LE would present several advantages. Firstly, less antibody would be required to ensure maximal binding of the BLE used in the assay and consequent low background fluorescence. Secondly, less LE would be required to displace sufficient BLE from the antibody to produce a measurable signal on interaction with FITC-avidin and therefore, LE concentrations in the assay displacing the maximum amount of BLE may not reach the concentrations which were observed to produce a direct interaction with FITC-avidin and as a result interfered with the success of the assay. Thirdly, since lower concentrations of LE would be required to displace BLE from the antibody and produce a measurable signal, the sensitivity of the assay would be increased.

A fluorescence spectrophotometer set at close to maximum signal amplification was used to measure the fluorescence signal in the development of the homogenous fluorescence immunoassay. The use of laser-induced fluorescence may have allowed the successful development of the proposed homogeneous immunoassay as less BLE could have interacted with a lower concentration of FITC-avidin to produce a measurable signal. Therefore, less LE would have been required to displace BLE from antibody binding sites so that the higher concentrations of LE which were observed to produce a direct interaction with FITC-avidin would not have been attained.

In conclusion, although it has been demonstrated that the homogenous fluorescence immunoassay for LE proposed in this study works in theory, a fully operational assay could not be developed in practice as the concentrations of LE required in this assay to displace the maximum amount of BLE from the antibody interacted directly with FITC-avidin to produce fluorescence enhancement of the detector molecule. Existing homogeneous fluorescence immunoassays for haptens and proteins lie in the 10^{-8} to 10^{-9} moles/ml range [Jenkins 1992] and homogeneous enzyme immunoassays are capable of limits of detection 10^{-14} moles/ml of haptens [Engel and Khanna 1992]. It is proposed that the use of an antibody with higher affinity for LE would allow the successful development of the type of homogeneous fluorescence immunoassay for LE described in this study as lower concentrations of LE could be used and therefore the direct interaction between LE and FITC-avidin that was encountered would be avoided. It is anticipated that the successful development of this type of homogeneous immunoassay would allow the detection of LE in the 10^{-9} to 10^{-11} moles/ml range.

CHAPTER 3
A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR OPIOID PEPTIDES USING ELECTROCHEMICAL DETECTION

Introduction

The objective of this study was the evaluation of a tyrosine-specific clean-up and detection method for opioid peptides using leucine enkephalin (LE, Figure 3.1) as a model peptide. The assay described here is based on the derivatization of LE by specific enzymatic o-hydroxylation of the highly conserved tyrosine groups in the 1 position of opioid peptides by mushroom tyrosinase. This derivatization results in the formation of a catechol which is amenable to specific clean-up using boronate gels and is more easily oxidizable than the parent peptide, thus facilitating electrochemical detection.

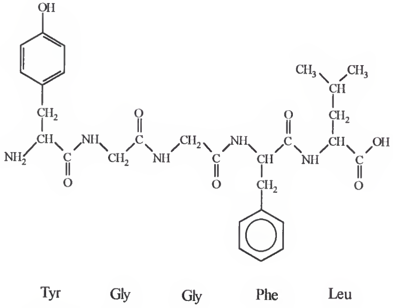


Figure 3.1. Leucine enkephalin

Mushroom tyrosinase is a member of the monoxygenase class of enzymes which catalyses two successive reactions (Figure 3.2): the hydroxylation of mono-phenols (monophenolase activity) and the oxidation of o-diphenols (diphenolase activity) [Walsh 1979]. The o-quinones resulting from these two successive reactions often form high molecular weight polymerization products in vivo such as melanin. In this study, the formation of undesirable polymerization products was prevented by the addition of appropriate amounts of ascorbic acid as a reductant.

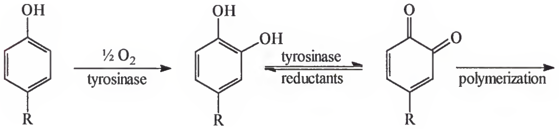


Figure 3.2. Tyrosinase catalyzed reactions

Small molecules such as L- and D-tyrosine and L- and D-dopa are endogenous substrates for tyrosinase. However, enzyme activity has been shown with tyrosine-containing di- and tri-peptides [Marumo and Waite 1986, Tellier et al. 1991] as well as LE and ME [Rosei et al. 1991, Rosei et al. 1989]. Larger proteins such as insulin, serum albumin and dehydrogenase enzymes have also been shown to be oxidized by tyrosinase [Cory et al. 1962, Cory and Frieden 1967a, Cory and Frieden 1967b, Gemant 1974, Ito et al. 1984].

In the assay described here, the enzymatic derivatization of LE by the means described above presents two analytical advantages. Firstly, the specific o-hydroxylation of the tyrosine group of LE to give a more easily oxidizable catechol allows the use of a lower oxidation potentials for electrochemical detection, thus avoiding many of the disadvantages associated with high applied potentials such as high background current and baseline noise [Kim et al. 1989]. Selectivity is also compromised when high applied potentials are used as more compounds are oxidized at these high potentials. Therefore, extensive clean-up procedures to eliminate interfering peaks are often required when high applied potentials are used [Fleming and Reynolds 1988]. Secondly, the enzymatic derivatization increases the selectivity of this assay as the derivative is amenable to a specific boronate clean-up method which has previously been established for catecholamines [Eriksson and Wikstrom 1992, Higa et al. 1977, Koike et al. 1982].

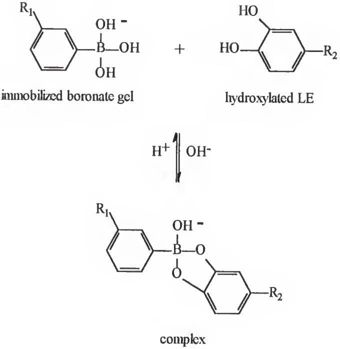


Figure 3.3. pH dependent complex formation between immobilized boronate gel and hydroxylated LE.

The introduction of a 3-hydroxytyrosine group to the LE molecule by enzymatic derivatization allows the use of a specific clean-up procedure for 3,4-dihydroxyphenyl compounds using boronate gels and column chromatography. For this assay, the boronate clean-up method is based on the pH-dependent formation of a complex between immobilized boronate gel and the hydroxylated LE derivative (Figure 3.3). A complex is formed at weakly alkaline pH between ionized boronate affixed to a gel matrix and the hydroxylated LE derivative. Dissociation of the complex occurs at acidic pH.

Therefore, in the assay described here, LE in the sample is first derivatized enzymatically by mushroom tyrosinase and subsequently, the sample is subjected to clean-up through the use of a boronate gel column. Finally, the sample is quantified by high performance liquid chromatography with electrochemical detection (HPLC-ED).

Materials

Leucine enkephalin and mushroom tyrosinase were obtained from Sigma Chemical Company, St. Louis, MO, USA. Acetonitrile, methanol and trifluoroacetic acid were of HPLC grade and disodium hydrogen phosphate and citric acid were of reagent grade. These chemicals were procured from Fisher Scientific, Pittsburgh, PA, USA. Sodium dihydrogen phosphate was of molecular biology grade and was purchased from Fluka Chemie, Buchs, Switzerland. All other chemicals were of reagent grade. Double distilled water was used throughout.

Methods

Purification of Mushroom Tyrosinase

Mushroom tyrosinase was purified prior to use by ultra-filtration using Centricon membrane filters (molecular weight cut off 30,000, Amicon, Danvers, MA, USA). One milliliter of a solution of mushroom tyrosinase (1 mg/ml) in 0.1 M sodium phosphate buffer pH 7 was applied to the filter unit and centrifuged at 5,000 g until maximum concentration of the sample was achieved. This centrifugation step was repeated three times with the addition of an additional 2 ml of phosphate buffer prior to each centrifugation. The final concentrate was reconstituted in phosphate buffer to give a final concentration of 1 mg/ml of mushroom tyrosinase corresponding to 3870 units of activity per ml of solution. Aliquots were stored at -20°C and defrosted immediately prior to use.

Enzymatic Derivatization

To characterize the enzymatic derivatization procedure, the following incubation mixture was set up in a microcentrifuge tube: 1 mM LE, 135 units/ml mushroom tyrosinase and 50 mM ascorbic acid in 0.5 M phosphate buffer pH 7.4. The reaction was allowed to proceed at room temperature with constant shaking for 60 minutes. Aliquots of this incubation mixture were then applied to an HPLC system consisting of an LDC/Milton Roy miniMetric II metering pump (Riviera Beach, FL, USA), a Negretti and Zamba injector (Southampton, UK) fitted with a 500 µl loop, a Perkin Elmer LC-75 spectrophotometric detector (Norwalk, CT, USA) and a Hewlett Packard HP 3394A integrator (Avondale, PA, USA). The column was a Partisil 5 ODS-3 125 x 4.6 mm (Whatman Labsales, Hillsboro, OR, USA). The detection wavelength was 254 nm and the mobile phase consisted of 12.5% acetonitrile (v/v) in citrate/dipotassium phosphate buffer (pH 5) at a flow rate of 1 ml/minute.

The product peaks were collected from the HPLC eluent, organic solvent was removed by evaporating under a stream of nitrogen and the collected peaks were concentrated using a preconditioned Sep-Pak C_{18} preparative column (Waters Associates, Milford, MA, USA). The identity of the products was determined by electrospray ionization mass spectrometry (see Chapter 5).

Electrochemical Detection

Electrochemical detection was effected using a Model 5100A Coulochem multi-electrode electrochemical detector (ESA Inc., Bedford, MA, USA) fitted with a Model 5020 guard cell and a Model 5011 analytical cell. The complete HPLC-ED system was configured as shown in Figure 3.4.

In this system, the guard cell acts to pre-oxidize electroactive impurities in the mobile phase, thus reducing background current. The analytical cell consists of two working electrodes in series. The first (Det 1) acts to further reduce background current in the injected sample and to pre-oxidize co-eluting interferences that oxidize at potentials lower than the analyte of interest. The second working electrode (Det 2) is set to quantify the analyte. The solvent delivery system was an LDC/Milton Roy constaMetric III metering pump (Riviera Beach, FL, USA), and the injector was a Rheodyne Model 7125 injector (Cotati, CA, USA) fitted with a 100 µl loop. A Spherisorb ODS2 5 µm 150 x 4.6 mm analytical column (Keystone Scientific Inc., Bellefonte, PA, USA) was used with a mobile phase of 20% acetonitrile (v/v) in monosodium phosphate buffer (100 mM, pH 5) containing 200 mg/l of sodium dodecyl sulfate. The mobile phase was freshly prepared, filtered through a 0.2 µm membrane filter and degassed under vacuum with sonication daily, prior to use. Mobile phase flow rate was set at 1 ml/min. A Chromatopac C-R3A integrator (Shimadzu Corporation, Kyoto, Japan) was used to record the output from the control module.

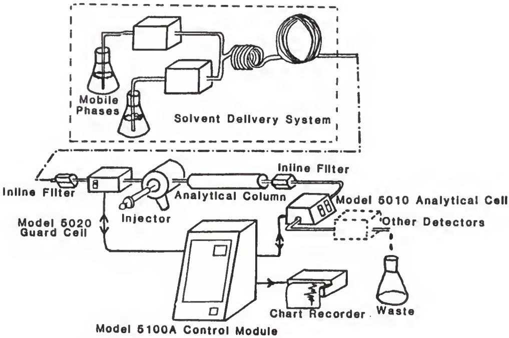


Figure 3.4. Chromatographic configuration of electrochemical detection system.

Electrochemical Characterization

LE and monohydroxylated leucine enkephalin ([HO-Tyr[1]]-LE), the major product of the enzymatic reaction were characterized electrochemically using the HPLC-ED system described above. Five nanomoles of analyte per injection were applied to the system and the responses (in μA) obtained at various potentials (+0.05 to +0.80V) at the analytical cell were recorded to allow the construction of current-voltage curves.

Boronate Clean-up

A hydrated boronate column packed with a 3 ml bed volume of Affigel 601 (BioRad Laboratories, Melville, NY, USA) was used for the clean-up of the enzymatically derived species prior to application to the HPLC-ED system. The column was pre-rinsed with 0.2 M phosphate buffer (pH 8.5) and an aliquot of the incubation mixture was applied. The column was washed with 20 ml of phosphate buffer (pH 8.5) and the analyte was eluted from the boronate column onto a pre-conditioned Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA, USA) with 20 ml of aqueous 0.01% (v/v) trifluoroacetic acid (TFA). Here, the Sep-Pak C_{18} cartridge served to concentrate the sample as the analyte was eluted from the boronate column in a relatively large volume of aqueous 0.01% (v/v) TFA The Sep-Pak C_{18} column was then washed with 10 ml of aqueous 0.01% (v/v) TFA and the analyte was eluted in 2 ml of 0.01% TFA in acetonitrile. The sample was evaporated to dryness under a stream of nitrogen and the residue was reconstituted in 200 μl mobile phase prior to application to the HPLC-ED system.

Time Course of Enzymatic Derivatization

To determine the time course of the enzymatic derivatization at the analytical concentrations, the following incubation mixture was set up in microcentrifuge tubes: $8*10^{-8}$ M LE, 50 mM ascorbic acid and 135 units/ml mushroom tyrosinase in 300 μl of 0.5 M phosphate buffer pH 7.4. The reaction was stopped at various time points by adding 20 μl of 1 N HCl. Two hundred an fifty micrometers of this incubation mixture was then subjected to the boronate clean-up procedure, the resulting sample was injected into the HPLC-ED system and the peak areas of the peak corresponding to the enzymatic derivative [HO-Tyr^1]-LE were recorded.

Extraction of Leucine Enkephalin from Cerebrospinal Fluid

Leucine enkephalin was extracted from human cerebrospinal fluid (CSF) through the use of Supelclean LC 18 solid phase extraction columns (Supelco Inc., Bellefonte, PA, USA). The columns were activated with 3 ml each of water and methanol and loaded with 100 μl of spiked CSF. Subsequently, the columns were washed with 1 ml of water, 3 ml of 0.1 N HCl, 1 ml of water, 3 ml of 0.1 M borate buffer (pH 8.5) and 1 ml of water. The LE rich fraction was then eluted in 2 ml of methanol and evaporated to dryness under a stream of nitrogen.

Calibration Curves

Using the complete HPLC-ED method, including enzymatic derivatization and boronate clean-up, calibration curves were constructed for LE in both buffer and CSF. For comparison, calibration curves for LE in CSF were also constructed using HPLC-ED without enzymatic derivatization or boronate clean-up.

Results

Enzymatic Derivatization

Preliminary experiments indicated that the concentration of mushroom tyrosinase (135 units/ml) used for the enzymatic derivatization of LE allowed the efficient conversion of LE to its hydroxylated derivative within 60 minutes (data not shown). A 50 mM concentration of ascorbic acid was found to be adequate to prevent or reverse the tyrosinase-induced formation of o-quinones in the enzymatic reaction mixture, thereby inhibiting the subsequent polymerization of the reaction products. Figure 3.5A shows the chromatograph of a control run where only LE and ascorbic acid are present at the incubation concentrations. Here, LE is seen eluting after 9.5 minutes, distinct from ascorbic acid eluting in the solvent front after 1.4 minutes. Two additional peaks at 5.7 minutes and 7.3 minutes are seen in the chromatograph of the incubation solution (Figure 3.5B). These two product peaks were identified by electrospray ionization mass spectroscopy (see Chapter 5) as the di- and mono-hydroxylated derivatives of LE ([$(HO)_2$- Tyr^1]-LE and [HO-Tyr^1]-LE, respectively). The relative intensity of the peaks obtained from mass spectrometric analysis showed that [$(HO)_2$-Tyr^1]-LE is a minor product.

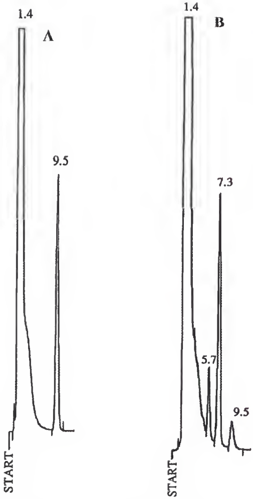


Figure 3.5. Chromatographs of a control run (A) showing LE eluting after 9.5 minutes, distinct from ascorbic acid eluting in the solvent front after 1.4 minutes and incubation solution (B) showing the emergence of two new peaks with retention times of 5.7 and 7.3 minutes.

Electrochemical Characterization

Current-voltage curves for LE and [HO-Tyr^1]-LE are shown in Figure 3.6. When these curves were compared, [HO-Tyr^1]-LE was seen to be oxidized at considerably lower potentials than LE. As a result of this experiment, the following potentials were selected for use in the construction of calibration curves for LE using the complete HPLC-ED method (i.e. including enzymatic derivatization and boronate clean-up) and HPLC-ED without enzymatic derivatization or boronate clean-up:

	HPLC-ED with derivatization and cleanup	HPLC-ED, no derivatization, no cleanup
Guard cell	+0.4 V	+0.75 V
Analytical cell Det 1	-0.1 V	+0.4 V
Analytical cell Det 2	+0.3 V	+0.7 V

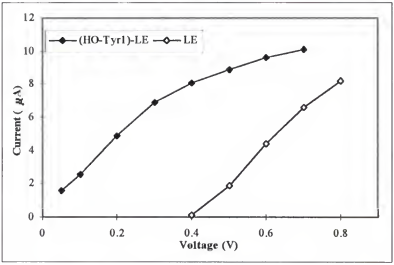


Figure 3.6. Current-voltage curves for (HO-Tyr^1)-LE and LE

Using these potentials, impurities in the mobile phase are pre-oxidized by the guard cell thus reducing background current and baseline noise. Impurities in the sample with relatively low oxidation potentials which might co-elute with the analyte and interfere with the signal produced by the analyte are preoxidized at Det 1. The Det 1 potential is set sufficiently low so that the analyte will not be pre-oxidized at this electrode, thus ensuring the production of a maximum signal at the analytical potential at Det 2.

Boronate Clean-up

By applying 35 pmoles to the boronate gel column, an average recovery of analyte of 68.67 % was achieved using the complete boronate clean-up method (SD = 6.1, n = 3).

Time Course of Enzymatic Derivatization

A representative time course of the enzymatic derivatization at analytical concentrations (17 pmol/inj) showing the peak area of [HO-Tyr^1]-LE plotted against time is shown in Figure 3.7. This experiment was carried out twice, showing the same trend each time. The plot indicates that the highest level of [HO-Tyr^1]-LE is seen after a 5 minute incubation and therefore, this incubation time was selected for future use in this study.

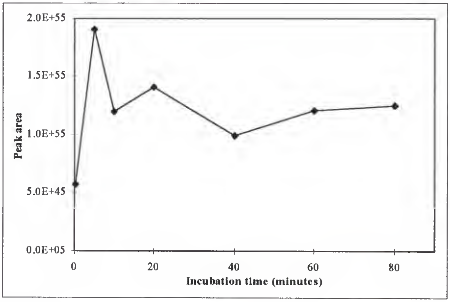


Figure 3.7. Time course of enzymatic derivatization showing peak height of [HO-Tyr^1]-LE over incubation time.

Calibration Curves

Table 3.1 shows the limits of detection (LOD, defined as twice the baseline noise), average slope values and correlation coefficients (r^2) obtained for the various calibration curves constructed for LE using this analytical approach. Raw data can be found in Appendix A. Representative calibration curves for LE in buffer and CSF using the complete HPLC-ED method are shown in Figures 3.8 and 3.9. Figure 3.10 shows a representative calibration curve for LE in CSF using HPLC-ED without enzymatic derivatization or boronate clean-up. The limits of detection for LE in CSF correspond to 8.8 pmol/ml of CSF for the complete HPLC-ED method and 176 pmol/ml of CSF for analysis by HPLC-ED without derivatization or boronate clean-up. A sample chromatograph of LE in CSF shows the analyte eluting after 6 minutes (Figure 3.11).

Table 3.1. Limits of detection (LOD), average slopes and correlation coefficients (r^2) for LE in buffer or CSF using either complete HPLC-ED method or HPLC-ED without derivatization or boronate clean-up. For raw data, see Appendix A.

Sample	Method	LOD fmol/inj	Slope (SD, n) peak area/pmol/inj	r^2 (SD, n)
Buffer	complete HPLC-ED	170	28071 (2381, 3)	0.9925 (0.005, 3)
CSF	complete HPLC-ED	360	22057 (1064, 3)	0.9944 (0.004, 3)
CSF	HPLC-ED no derivatization no clean-up	8800	10217 (1163, 3)	0.9690 (0.024, 3)

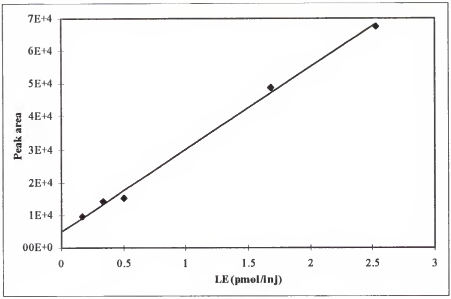


Figure 3.8. Representative calibration curve for LE in buffer using complete HPLC-ED method.

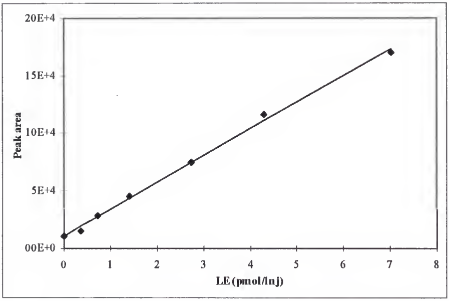


Figure 3.9. Representative calibration curve for LE in CSF using complete HPLC-ED method.

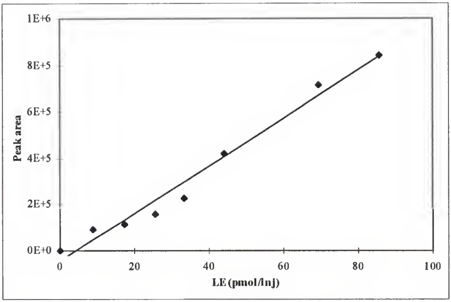


Figure 3.10. Representative calibration curve for LE in CSF using HPLC-ED without derivatization or boronate clean-up.

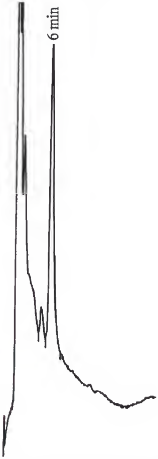


Figure 3.11. Sample chromatograph of LE in CSF showing peak of interest eluting after 6 minutes.

To give an indication of the inter-day variability associated with the complete HPLC-ED method incorporating enzymatic derivatization and boronate clean up, using the calibration curves obtained for LE in CSF, nominal concentrations in the samples were compared to found concentrations (Table 3.2). The found concentrations were determined from the calibration curve after regression analysis was repeated while omitting the data point under investigation. The relative standard deviation of the found concentrations was found to be ≤20% (n=3) and the accuracy was found to be within 6% of the nominal concentration.

Table 3.2. Nominal concentrations, found concentrations, relative standard deviation (SD) and percentage accuracy calculated from 3 calibration curves for LE in CSF using complete HPLC-ED method. For raw data, see Appendix A.

Nominal conc. (pmol/100 µl CSF)	Found conc, n=3 (pmol/100 µl CSF)	Relative SD (%)	% Accuracy
1.76	1.84	20	104
3.46	3.34	12	97
6.67	6.41	12	96
10.48	11.13	1	106
17.14	17.65	9	103

Discussion

The HPLC-ED method we have evaluated for the analysis of LE in CSF compares favorably to existing HPLC methods for opioid peptides with on-line detection [de Montigny et al. 1990, Mifune et al. 1989, Muck and Henion 1989] and represent an improvement with respect to limit of detection and practicability when compared to current HPLC-ED methods for enkephalins (Table 3.3) [Fleming and Reynolds 1988, Kim et al. 1989, Shibanoki et al. 1990]. The increased practicability of the analytical approach described here is characterized by the minimal precautions required in the preparation of the mobile phase (filtering and degassing under negative pressure with sonication) and is due to the fact that the enzymatic derivatization employed allowed the use of lower applied potentials for electrochemical detection (+0.3 V compared to +0.85-1.25 V for existing HPLC-ED methods). The low applied potentials used allowed the electrochemical detector to be operated at the maximum gain setting since background current and baseline noise were low. These factors, in addition to the minimal baseline drift observed, increased the ease of handling of this analytical approach since minimal precautions could be taken in the preparation of mobile phase and samples. This was demonstrated when a comparison was made between the analysis of LE in CSF using the complete HPLC-ED method incorporating enzymatic derivatization and boronate clean up and the HPLC-ED analysis of LE in CSF without derivatization or boronate clean-up. When an attempt was made to analyze LE in CSF without derivatization or boronate clean-up, considerably higher applied potentials had to be used (+0.7 V compared to +0.3 V) and as a consequence, background current, baseline noise and baseline drift were greatly increased. Therefore, the maximum gain setting of the instrument could not be used, sensitivity was compromised by a factor of 25 and the linearity of the calibration curves was reduced. In addition, the system was more difficult to handle as the mobile phase had to be continually degassed under a stream of helium in order to avoid unacceptable baseline drift.

Table 3.3. Comparison table of current analytical methods for opioid peptides.

Reference	Method	Analyte	Matrix	Sensitivity
This study	HPLC-ED	LE	Buffer	170 fmol/inj
This study	HPLC-ED	LE	CSF	360 fmol/inj
Fleming and Reynolds 1988	HPLC-ED	enkephalin	rat brain	1 pmol/inj
Shibanoki et al. 1990	HPLC-ED	enkephalin	plasma	550 fmol/inj
Kim et al. 1989	HPLC-ED	enkephalin	rat brain	1 pmol/inj
Muck and Henion 1989	HPLC-MS	dynorphin	CSF	100 fmol/inj
Mifune et al. 1989	HPLC-FL	enkephalin	rat brain	100 fmol/inj
de Montigny et al. 1990	HPLC-FL	LE	plasma	7.7 pmol/inj

HPLC-MS = high performance liquid chromatography with mass spectrometry, HPLC-FL = high performance liquid chromatography with fluorescence detection.

The boronate clean-up procedure used in the HPLC-ED assay described here proved to be an effective and efficient clean-up method for LE in CSF resulting in a clean chromatograph for the analyte (Figure 3.11). To avoid interfering peaks, existing HPLC-ED methods for enkephalins involve complex and relatively non-selective sample clean up procedures (e.g./ multiple precipitation, centrifugation and adsorption steps) prior to application of the sample to the HPLC system. By contrast, here the use of a boronate clean-up procedure, which had been previously established for use with catecholamines [Higa et al. 1977, Koike et al. 1982], increased the selectivity of this assay as only derivatized peptide incorporating the dihydroxy group introduced by enzymatic derivatization was retained on the boronate gel column. The limits of detection achieved for LE using this analytical approach (170 fmol/inj in buffer and 360 fmol/inj in CSF) also compared favorably to assays for catecholamines using a boronate clean-up method and electrochemical detection (≈200 fmol/inj) although the recovery of hydroxylated LE from the boronate gel (69%) was considerably lower than the recovery of catecholamines from the same matrix (80-100%) [Higa et al. 1977, Koike et al. 1982].

The HPLC-ED approach for the determination of LE in CSF described here was found to be reproducible and accurate since the relative standard deviations of various concentrations determined on different days was found to be $\leq$ 20% and found concentrations were determined to be within 6% of nominal concentrations (Table 3.2).

In theory, the analytical method we have described here is applicable to a whole range of opioid peptides since the N-terminal tyrosine group which is derivatized is highly conserved throughout the entire family of opioid peptides. This method should also be applicable to the analysis of other tyrosine-containing proteins and peptides as it has been shown previously that the amino acid adjacent to the tyrosine group does not dramatically influence the tyrosinase reaction [Tellier et al. 1991]. Endogenous levels of opioid peptides in human CSF lie in the fmol/ml range as determined by radioimmunoassay [Eisenach et al. 1990, Hardebo et al. 1989, Samuelsson et al. 1993, Yaksh et al. 1990, Young et al. 1993]. Although the analytical approach described here is inadequate for the determination of endogenous levels of opioid peptides in human CSF, given sufficient sample (>1 ml), it may be adequate for the analysis of the elevated physiological concentrations of opioid peptides to be expected in clinical studies.

CHAPTER 4
A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ASSAY FOR OPIOID PEPTIDES USING FLUORESCENCE DETECTION

Introduction

The objective of this study was the evaluation of a tyrosine-specific analytical approach for opioid peptides using high performance liquid chromatography with fluorescence detection and leucine enkephalin (LE, Figure 4.1) as a model peptide.

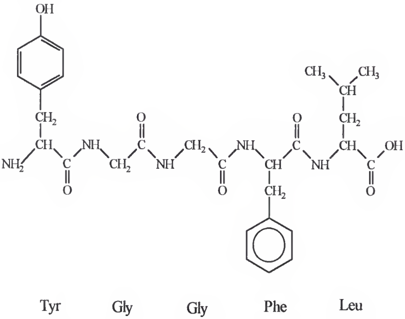


Figure 4.1. Leucine enkephalin

As in Chapter 3, this analytical approach exploits the derivatization of LE by specific enzymatic o-hydroxylation of the highly conserved tyrosine groups in the 1 position of opioid peptides by mushroom tyrosinase. Here, the enzymatic derivatization renders the peptide suitable for subsequent fluorogenic derivatization using 1,2-diamino-1,2-diphenylethane (DPE). Therefore, in this assay, the hydroxylated LE derivative obtained from the reaction with mushroom tyrosinase is oxidized in a controlled manner by potassium ferricyanide to give the corresponding quinone prior to a condensation reaction with DPE to give a fluorescent product (Figure 4.2).

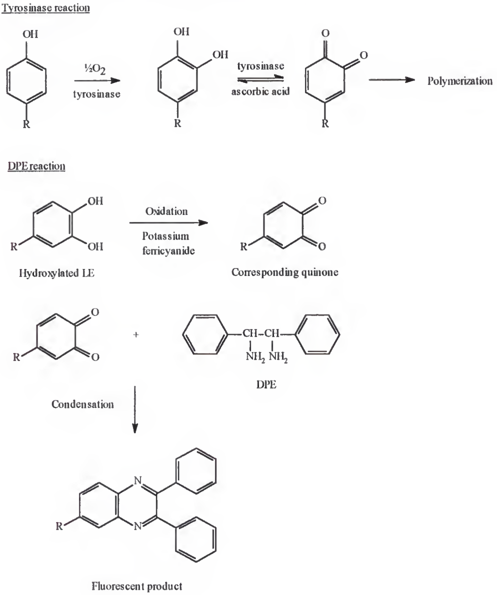


Figure 4.2. Overview of derivatization reactions for HPLC-FL assay

Materials

Leucine enkephalin and mushroom tyrosinase were obtained from Sigma Chemical Company, St. Louis, MO, USA. Acetonitrile, methanol and trifluoroacetic acid were of HPLC grade and disodium hydrogen phosphate, sodium dihydrogen phosphate, citric acid, potassium chloride and potassium ferricyanide were of reagent grade. These chemicals as well as Scintiverse II scintillation cocktail were procured from Fisher Scientific, Pittsburgh, PA, USA. Tetrabutylammonium (TBA) was purchased from the Eastman Kodak Company, Rochester, NY, USA and [tyrosyl-3,5-^{3}H(N)]-leucine enkephalin (^{3}H-LE) was obtained from NEN Research Products, Dupont Company, Wilmington, DE, USA. 1,2-Diamino-1,2-diphenylethane (DPE) was synthesized according to Irving and Parkins [Irving and Parkins 1965]. Briefly, benzaldehyde (1.9 equivalents) was refluxed with ammonium acetate (1 equivalent) for 3 hours, the resulting precipitate was collected, and washed with ethanol. This precipitate was then hydrolyzed with 33 % v/v H_2SO_4, benzoic acid and benzaldehyde were removed by steam distillation, and DPE was precipitated by neutralization with ammonium hydroxide. After recrystallization from petroleum ether, the product had a melting point of 118-119°C (literature, 120°C) [Irving and Parkins 1965] and a ^{1}H nuclear magnetic resonance spectrum which supported the assigned structure. All other chemicals were of reagent grade. Double distilled water was used throughout.

Methods

Purification of Mushroom Tyrosinase

Mushroom tyrosinase was purified prior to use by ultra-filtration using Centricon membrane filters (molecular weight cut off 30,000, Amicon, Danvers, MA, USA). One milliliter of a solution of mushroom tyrosinase (1 mg/ml) in 0.1 M phosphate buffer pH 7 was applied to the filter unit and centrifuged at 5,000 g until maximum concentration of the sample was achieved. This centrifugation step was repeated three times with the addition of an additional 2 ml of phosphate buffer prior to each centrifugation. The final concentrate was reconstituted in phosphate buffer to give a final concentration of 1 mg/ml of mushroom tyrosinase corresponding to 3870 units of activity per ml of solution. Aliquots were stored at -20°C and defrosted immediately prior to use.

Enzymatic Derivatization

To characterize the derivatization procedure, LE (1 mM) in 0.5 M phosphate buffer pH 7.4 was reacted with mushroom tyrosinase (135 units/ml) in the presence of ascorbic acid (50 mM) at room temperature with constant shaking. The product peaks obtained from this enzymatic derivatization were collected from the HPLC eluent, organic solvent was removed by evaporation under a stream of nitrogen and the collected peaks were concentrated using a Sep-Pak C_{18} preparative column (Waters Associates, Milford, MA, USA). The identity of the products was determined by electrospray ionization mass spectrometry (see Chapter 5).

To determine the time course of the reaction, at various time points, aliquots of this incubation solution were applied to an HPLC system consisting of an LDC/Milton Roy miniMetric II metering pump (Riviera Beach, FL, USA), a Negretti and Zamba injector (Southampton, UK) fitted with a 500 µl loop, a Perkin Elmer LC-75 spectrophotometric detector (Norwalk, CT, USA) and a Hewlett Packard HP 3394A integrator (Avondale, PA, USA). The column was a Partisil 5 ODS-3 125 x 4.6 mm (Whatman Labsales, Hillsboro, OR, USA). The detection wavelength was 254 nm and the mobile phase consisted of 12.5% acetonitrile (v/v) in citrate/dipotassium phosphate buffer (pH 5) at a flow rate of 1 ml/minute.

Extraction of Leucine Enkephalin from Cerebrospinal Fluid

Leucine enkephalin was extracted from human cerebrospinal fluid (CSF) through the use of Supelclean LC_{18} solid phase extraction columns (Supelco Inc., Bellefonte, PA, USA). The columns were activated with 3 ml each of water and methanol and loaded with 100 µl of spiked CSF. Subsequently, the columns were washed with 1 ml of water, 3 ml of 0.1 N HCl, 1 ml of water, 3 ml of 0.1 M borate buffer (pH 8.5) and 1 ml of water. The LE-rich fraction was then eluted in 2 ml of methanol and evaporated to dryness under a stream of nitrogen.

To test the recovery of LE from CSF using this extraction procedure, 100 µl of CSF spiked with ^{3}H-LE (17,300 counts per minute (CPM)) was applied to an extraction column and the procedure described above was carried out. The final methanolic LE-rich fraction was collected in 1 ml aliquots, 4 ml of Scintiverse II scintillation cocktail were added and the radioactivity (CPM) was measured using a Beckman LS 5,000 TD scintillation counter (Fullerton, CA, USA). As a control, the radioactivity in a sample containing the same amount of ^{3}H-LE as the spiked CSF in 1 ml of methanol was also measured.

Calibration curves

Calibration curves for LE (0.5-7 pmol/inj) in both buffer and CSF were constructed using this HPLC-FL analytical approach.

For the tyrosinase reaction, various concentrations of LE in 0.5 M sodium phosphate buffer pH 7.4 or spiked CSF extracts reconstituted in 0.5 M sodium phosphate buffer pH 7.4 were reacted with mushroom tyrosinase (135 units/ml) in the presence of ascorbic acid (50 mM). The total volume of this incubation mixture was 100 μl. After a 60 minute incubation at room temperature with constant shaking, 449 μl of an oxidizing solution containing 9.45 mg/ml potassium ferricyanide, 17.85 mg/ml potassium chloride and 55 % acetonitrile (v/v) was added followed by 45.5 μl of a solution of DPE containing 20 mg/ml in 0.1 N HCl. The fluorogenic reaction was allowed to proceed in the dark for 60 minutes at room temperature with constant shaking. A 250 μl aliquot of this reaction mixture, representing 42 % of the total final reaction mixture, was then injected into an HPLC system consisting of an LDC/Milton Roy miniMetric II metering pump (Riviera Beach, FL, USA), a Rheodyne Model 7125 injector (Cotati, CA, USA) fitted with a 500 μl loop, a Nucleosil C_{18} column (5μm, 150 x 4.6 mm, Keystone Scientific, Bellefonte, PA, USA) and a Perkin Elmer 650S spectrofluorodetector (Norwalk, CT, USA) set at λ_{ex} 345 nm and λ_{em} 480 nm with slit widths of 12.5 nm. A BD 41 chart recorder (Kipp and Zonen, Delft, The Netherlands) and a Hewlett Packard HP 3394A integrator (Avondale, PA, USA) recorded the output from the spectrofluorodetector. The mobile phase contained 32 % acetonitrile (v/v) and 67 mM TBA in citrate/dipotassium phosphate buffer (pH 5). The system was operated at a mobile phase flow rate of 1 ml/min.

An attempt was made to optimize the fluorogenic derivatization reaction by concentrating the fluorogenic reagents so that 100 µl of an oxidizing solution containing 40.1 mg/ml of potassium ferricyanide and 75.8 mg/ml of potassium chloride, 100 µl of acetonitrile and 25 µl of a solution of DPE containing 20 mg/ml in 0.1 N HCl were added to the buffer samples or the CSF samples reconstituted in buffer. After a 60 minute incubation in the dark with constant shaking, a 250 µl aliquot of this mixture, representing 77 % of the total final reaction mixture, was injected into the HPLC system described above.

Since experiments conducted in parallel indicated that at low concentrations of analyte, the highest yield of enzymatically derived species was obtained after a 5 minute tyrosinase incubation time (see Chapter 3), an attempt was also made to optimize the derivatization reaction by reducing the tyrosinase incubation time to 5 minutes.

Results

Enzymatic Derivatization

Preliminary experiments indicated that the concentration of mushroom tyrosinase (135 units/ml) used for the enzymatic derivatization of LE allowed the efficient conversion of LE to its hydroxylated derivative within 60 minutes. A 50 mM concentration of ascorbic acid was found to be adequate to prevent or reverse the tyrosinase-induced formation of o-quinones in our enzymatic reaction mixture, thereby inhibiting the subsequent polymerization of the reaction products.

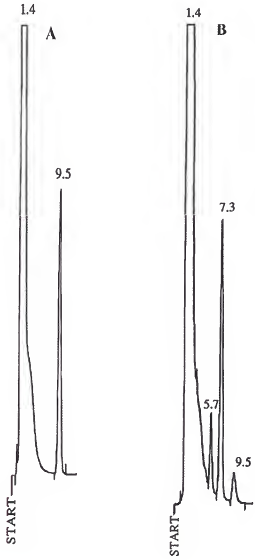


Figure 4.3. Chromatographs of a control run (A) showing LE eluting after 9.5 minutes, distinct from ascorbic acid eluting in the solvent front after 1.4 minutes and incubation solution (B) showing the emergence of two new peaks with retention times of 5.7 and 7.3 minutes.

Figure 4.3A shows the chromatograph of a control run where only LE and ascorbic acid are present at the incubation concentrations. Here, LE is seen eluting after 9.5 minutes, distinct from ascorbic acid eluting in the solvent front after 1.4 minutes. Two additional peaks at 5.7 minutes and 7.3 minutes are seen in the chromatograph of the incubation solution (Figure 4.3B). These two product peaks were identified by electrospray ionization mass spectroscopy (see Chapter 5) as the di- and mono-hydroxylated derivatives of LE ([$(HO)_2$- Tyr[1]]-LE and [HO-Tyr[1]]-LE, respectively).

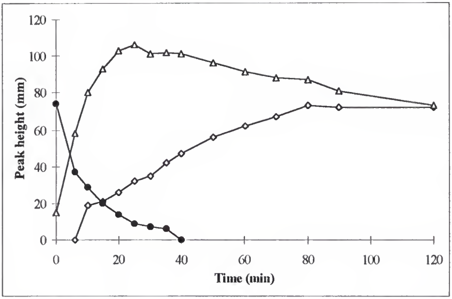


Figure 4.4. Time course for the enzymatic derivatization of LE (1 mM) by mushroom tyrosinase (135 units/ml) in the presence of ascorbic acid (50 mM) showing the disappearance of LE (•) as [HO-Tyr[1]]-LE (Δ) and $(HO)_2$-Tyr[1] (◊) are produced.

A time course of the enzymatic derivatization of LE by mushroom tyrosinase in the presence of ascorbic acid is shown in Figure 4.4. This time course shows the disappearance of the peak corresponding to LE as [HO-Tyr[1]]-LE and [$(HO)_2$- Tyr[1]]-LE are produced. An incubation time of 60 minutes was chosen for the enzymatic derivatization in our HPLC-FL assay as at this time point, the peak corresponding to LE had disappeared and the peak corresponding to our enzymatically derived species of interest ([HO-Tyr^1]-LE) was seen to plateau. This time course was repeated several times and the same trend was observed each time.

Recovery of Leucine Enkephalin from Cerebrospinal Fluid

Recovery of LE from CSF was estimated at >90% when the counts per minute in the methanolic eluent of the extraction column following the extraction procedure was compared to the control sample.

Calibration Curves

For the fluorogenic reaction, the concentration of reagents used was the same as those used previously for fluorogenic derivatization of tyrosine-containing peptides following enzymatic hydroxylation by mushroom tyrosinase [Tellier et al. 1991]. Here, as before, the concentrations of DPE and KCl used were taken directly from corresponding catechol assays [Mitsui et al. 1985, Nohta et al. 1984] but the concentration of potassium ferricyanide was increased to ensure the formation of the quinone from the hydroxylated LE derivative despite the presence of high concentrations of ascorbic acid. Acetonitrile is included in the reaction mixture to facilitate the condensation reaction by reducing the thermodynamic activity of water.

Table 4.1 shows a summary of the results obtained using the various modifications attempted in our HPLC-FL assay. Some of the results using the same method and the same matrix could not be averaged since the output from the fluorescence detector was monitored using different equipment (integrator or chart recorder).

Table 4.1. Summary of results for HPLC-FL assay. For raw data, see Appendix B.

Method	Matrix	Limit of detection	N	r^2	slope
Non-concentrated reagents	buffer	562 fmol/inj	1	0.9966	$1.79*10^{12}$ area/mol
Non-concentrated reagents	buffer	281 fmol/inj	2	0.9836-0.9971	$1.14\text{-}3.40*10^{13}$ mm/mol
Non-concentrated reagents	CSF	500 fmol/inj	1	0.9975	$3.89*10^{12}$ area/mol
Non-concentrated reagents	CSF	891 fmol/inj	1	0.9670	$1.08*10^{13}$ mm/mol
Concentrated reagents	Buffer	500 fmol/inj	2	0.9926-0.9984	$2.25\text{-}5.09*10^{12}$ area/mol
Concentrated reagents	CSF	500 fmol/inj	3	0.9917±0.0061	$2.07\pm1.26*10^{12}$ area/mol
Concentrated reagents, 5 min tyrosinase	Buffer	277 fmol/inj	3	0.9895±0.0086	$6.69\pm3.80*10^{12}$ mm/mol

Figures 4.5 and 4.6 show representative calibration curves obtained for LE in buffer samples and spiked CSF, respectively, using the HPLC-FL method with non-concentrated reagents. Limits of detection for LE of 500 fmol/injection could be obtained

in both buffer samples and spiked CSF samples. This limit of detection corresponds to 12 pmol of LE per ml of CSF. Figure 4.7 shows a representative chromatograph for LE in CSF using our HPLC-FL method.

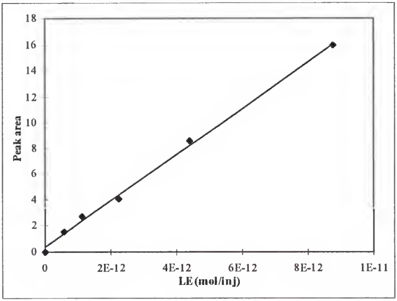


Figure 4.5. Calibration curve for LE in buffer using HPLC-FL method with non-concentrated reagents.

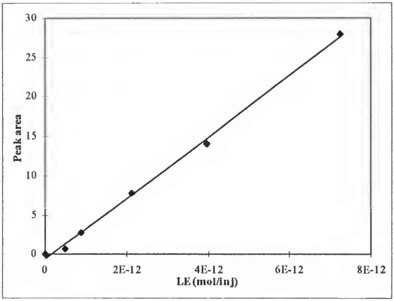


Figure 4.6. Calibration curve for LE in CSF using HPLC-FL method and non-concentrated reagents.

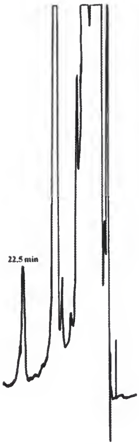


Figure 4.7. Chromatograph of LE in CSF using HPLC-FL method. LE is shown eluting after 22.5 minutes.

An analytical advantage was not gained through the concentration of the fluorogenic reagents and subsequent injection of a greater portion of the total final reaction mixture as the same limit of detection of 500 fmol/injection could be obtained when this modification in our method was attempted.

Although a lower limit of detection in buffer samples (277 fmol/injection) was obtained when a 5 minute reaction time instead of a 60 minute reaction time was used for the tyrosinase reaction (Figure 4.8), when this modification of the method was attempted in spiked CSF samples, the linearity of the calibration curve could not be preserved (data not shown). Attempts to improve the linearity of a calibration curve for LE in spiked CSF samples using a 5 minute tyrosinase reaction time by improving the extraction procedure or the incubation conditions were not pursued.

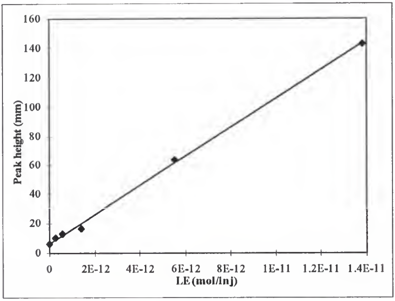


Figure 4.8. Calibration curve for LE in buffer samples using 5 minute tyrosinase reaction time in HPLC-FL method.

Discussion

The HPLC-FL analytical approach for LE developed in this study yielded detection limits of 500 fmol per injection in both buffer and spiked CSF samples, corresponding to 12 pmoles of LE per ml of CSF. This approach shows similar sensitivity or represents an improvement in the limit of detection by one order of magnitude when compared to existing tyrosine-specific HPLC-FL methods with pre-column fluorescence derivatization [Ishida et al. 1986, Kai et al. 1988, Nakano et al. 1987, Zhang et al. 1991]. The improvement in limit of detection seen with the approach described here may be attributed in part to the fact that in some of these existing tyrosine-specific HPLC-FL methods [Ishida et al. 1986, Kai et al. 1988], fluorogenic derivatization necessitates heating of the reaction mixture to 100°C for 3 minutes which may result in peptide instability and consequent reduced recovery of intact fluorescence-labeled analyte. Although more selective, the HPLC-FL approach described here does not achieve limits of detection for LE as low as those obtained by investigators using methods involving post-column fluorescence derivatization of the N-terminal primary amino group of opioid peptides (36-100 fmol/injection) [Dave et al. 1992, Mifune et al. 1989, van den Beld et al. 1990]. However, due to lack of selectivity, these methods often involve the use of extensive sample clean-up procedures and complex multi-dimensional chromatographic systems (column switching) to minimize the occurrence of interfering peaks [Mifune et al. 1989]. The higher sensitivity obtained using these methods can be attributed to the use of laser-induced fluorescence detection [Dave et al. 1992, van den Beld et al. 1990] and a microbore chromatographic system [Dave et al. 1992]. Incorporating a microbore chromatographic system and laser-induced fluorescence into the analytical approach described here could be expected to reduce considerably the limits of detection obtainable by this approach.

An attempt to improve the approached described in this study by concentrating the reagents in the fluorogenic reaction and thereby injecting a larger portion of the total reaction mixture did not result in an analytical advantage. This may be due to a decrease in the efficiency of the fluorogenic reaction in the presence of higher concentrations of fluorogenic reagents. Although a second attempt to improve the analytical approach by decreasing the tyrosinase reaction time resulted in a lower limit of detection in buffer samples, when this modification was attempted with spiked CSF samples, the linearity of the calibration curve was severely compromised. It is postulated that matrix components in the spiked CSF extracts may have altered the efficiency of the tyrosinase reaction, thus affecting the reproducibility of the yield of hydroxylated LE derivative after this short incubation time.

Table 4.2. Comparison table of HPLC-FL analytical methods for opioid peptides.

Reference	Sensitivity	Analyte	Matrix	Comments
This study	500 fmol/inj 12 pmol/ml CSF	LE	Buffer CSF	tyrosine specific
Ishida et al. 1986	7 pmol/inj	enkephalin	water	tyrosine specific
Nakano et al. 1987	270 fmol/inj	enkephalin	water	tyrosine specific
Kai et al. 1988	500 fmol/inj	enkephalin	rat brain	tyrosine specific
Zhang et al. 1991	0.33-1.21 pmol/inj	opioid peptides	rat brain	tyrosine specific
van den Beld et al. 1990	80 fmol/inj	β-endorphin	plasma	laser-induced fluorescence
Mifune et al. 1989	100 fmol/inj	enkephalin	rat brain	column-switching
Dave et al. 1992	36 fmol/inj	LE	water	microbore HPLC, laser-induced fluorescence

As with the HPLC-EC method described in Chapter 3, the HPLC-FL analytical approach described here should also be applicable to a whole range of opioid peptides since the N-terminal tyrosine group which is initially derivatized is highly conserved throughout the entire family of opioid peptides. This approach should also be applicable to the analysis of other tyrosine-containing proteins and peptides as it has been shown previously that the amino acid adjacent to the tyrosine group does not dramatically influence the tyrosinase reaction [Tellier et al. 1991]. Endogenous levels of opioid peptides in human CSF, as determined by radioimmunoassay lie in the fmol/ml range [Eisenach et al. 1990, Hardebo et al. 1989, Samuelsson et al. 1993, Yaksh et al. 1990, Young et al. 1993]. Therefore, here too, although this analytical approach in its present form is inadequate for the determination of endogenous levels of opioid peptides in human CSF, given sufficient sample (>1ml) it may also be adequate for the analysis of the elevated physiological concentrations of opioid peptides to be expected in clinical studies.

CHAPTER 5
LEUCINE ENKEPHALIN-TYROSINASE REACTION PRODUCTS - IDENTIFICATION AND BIOLOGICAL ACTIVITY

Introduction

Tyrosinase is a copper containing enzyme which catalyses the ortho-hydroxylation of phenols and the subsequent oxidation of the resulting catechols to o-quinones. It is common throughout nature and plays a central role in the biosynthesis of both norepinephrine and melanin. In Chapters 3 and 4 of this dissertation, we have shown that tyrosinase will react with the tyrosine-containing peptide leucine enkephalin (LE). Previously, our group and others have shown that tyrosinase also reacts with other tyrosine-containing peptides to give hydroxylated products [Marumo and Waite 1986, Rosei et al. 1991, Rosei et al. 1989, Tellier et al. 1991].

Numerous studies have revealed a loss of activity in enkephalins and other opioid peptides when the Tyr^1 moiety is absent (for reviews, see Hansen and Morgan 1984 and Shimohigashi 1986), however, few have shown the effect of modification of the aromatic side-chain of this residue. Therefore, as tyrosinase is known to react with tyrosine-containing peptides, our goal in this study was to determine the structure of the products formed when tyrosinase reacts with LE and to define the affinity of the products modified at the Tyr^1 residue to opioid receptors in rat brain homogenate.

Materials

The following materials were procured from the sources indicated: Leucine enkephalin, mushroom tyrosinase (3870 units/mg, E.C. 1.14.18.1), bestatin, thiorphan and captopril from Sigma, St. Louis, MO, USA, ascorbic acid from Mallinckrodt, Paris, KY, USA, [^{3}H]-diprenorphine from Amersham International, Arlington Heights, IL, USA, and Scintiverse II scintillation cocktail from Fisher Scientific, Pittsburgh, PA, USA. All other chemicals were of reagent grade. Double distilled water was used throughout.

The chromatographic system used consisted of an LDC/Milton Roy miniMetric II metering pump (Riviera Beach, FL, USA), a Negretti and Zamba injector (Southampton, UK) fitted with a 500 μl loop, a Perkin Elmer LC-75 spectrophotometric detector (Norwalk, CT, USA) and a Hewlett Packard HP 3394A integrator (Avondale, PA, USA). The column was a Partisil 5 ODS-3 125 x 4.6 mm (Whatman Labsales, Hillsboro, OR, USA). The detection wavelength was 254 nm and the mobile phase consisted of 12.5% acetonitrile (v/v) in citrate/dipotassium phosphate buffer (pH 5) at a flow rate of 1 ml/minute.

Methods

Enzymatic Reaction

The reaction between LE and mushroom tyrosinase was carried out in microcentrifuge tubes at the following concentrations: 1 mM LE and 50 mM ascorbic acid in 0.1 M potassium phosphate buffer (pH 7). The reaction was started by adding mushroom tyrosinase (previously purified as described in Chapters 3 and 4) to give a final concentration of 135 units/ml. The reaction mixture was incubated at room temperature with constant shaking and the reaction was stopped after 1 hour by adding 66 µl of 1N HCl per ml of incubation solution. A control incubation was carried out in parallel, omitting the addition of mushroom tyrosinase to insure the integrity of LE under the reaction conditions.

Product Identification

For the isolation of the reaction products, a total of 4 ml of incubation solution was injected into the HPLC system in 500 µl aliquots. The product peaks were collected and pooled according to their retention times. The collected peaks were then concentrated under a stream of nitrogen to remove organic solvents and prepared for analysis by mass spectrometry as follows: A Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA, USA) was activated with 2 ml of methanol and washed with 3 ml each of methanol/3% acetic acid 70/30 v/v and 3% acetic acid v/v. The concentrated materials were applied to the cartridge and the cartridge was washed with 3 ml of 3% acetic acid v/v. The analytes were then eluted in 500 µl of methanol/3% acetic acid 70/30 v/v and stored at -20°C prior to mass spectrometric analysis.

A Vestec 200ES instrument (Vestec Corp., Houston, TX, USA) was used to obtain the electrospray ionization (ESI) mass spectra [Allen and Vestal 1992]. The sample solution was drawn into a standard laboratory syringe (250 µl, Model 1710, Hamilton Co., Reno, Nevada, USA) and supplied into the electrospray probe through a 50 cm x 0.1 mm i.d. deactivated fused silica capillary (Scientific Glass Engineering, Victoria, Australia) at 2 to 5 μl/min flow rate by a medical infusion pump (SAGE Instruments, Model 341B, Boston, MA, USA). A 0.005 in i.d. x 0.010 in o.d. flat tipped hypodermic needle held at 2.4 kV potential produced spray current in the range of 130 to 180 μA, when the tip of the needle to nozzle orifice distance was about 10 mm. The source block was heated to 250 °C, and the spray chamber temperature was around 55 to 60 °C. A Vector/One data system (Teknivent, St. Louis, MO, USA) was used to control the quadrupole analyzer (2,000 Da mass range), and to collect mass spectra in the 100 to 1,000 Da mass range at 3 ms/Da scan rate. For molecular weight determination, the repeller to collimator voltage was held at 18 V, and collision-induced dissociation in the skimmer to collimator region was obtained at 50 V. At least ten spectra were averaged for each experiment.

Radioreceptor Assay

The enzymatic reaction and radioreceptor assay were carried out on the same day. Two hundred microliters of the incubation solution from the enzymatic reaction were injected into the HPLC system and the peak corresponding to [HO-Tyr^1]-LE was collected in 1.9 ml of mobile phase. A 90% conversion from LE to [HO-Tyr^1]-LE was assumed based on the disappearance of the peak corresponding to LE after a 1 hour incubation with mushroom tyrosinase (Figure 5.1) and the relative intensities of the product peaks obtained from mass spectrometric analysis. Therefore, this solution contained 180 nmoles of [HO-Tyr^1]-LE and was used directly in the radioreceptor assays. Two hundred microliters of a 1 mM solution of LE were also injected into the HPLC system and the peak corresponding to LE was collected and used in the radioreceptor assays. Mobile phase components were found not to interfere with the radioreceptor assay.

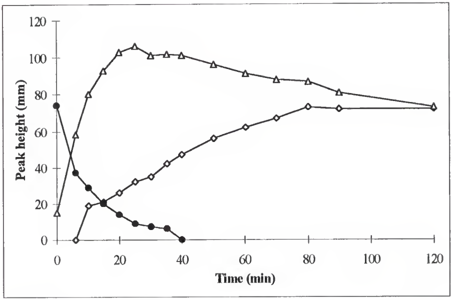


Figure 5.1. Time course for the enzymatic derivatization of LE (1 mM) by mushroom tyrosinase (135 units/ml) in the presence of ascorbic acid (50 mM) showing the disappearance of LE (•) as [HO-Tyr^1]-LE (Δ) and $(HO)_2$-Tyr^1 (◊) are produced.

Rat brain membranes were prepared as described by Hochhaus et al [Hochhaus et al. 1988]. Briefly, the whole brain, without cerebellum, of male Sprague-Dawley rats (120-140 g) was homogenized in 60 volumes of ice cold 50 mM Tris-HCl buffer (pH 7.4) containing 100 mM NaCl. The homogenate was incubated for 1 hour at 20°C and centrifuged for 20 minutes at 4°C. The pellet was then resuspended, washed twice with 50 mM Tris-HCl and diluted in 50 mM Tris-HCl to give 400 mg of rat brain membranes per ml. Aliquots of this suspension were stored at -80°C and used within one week.

The radioreceptor assays were carried out in duplicate in microcentrifuge tubes. Each tube contained 30 μM bestatin, 0.6 μM thiorphan and 10 μM captopril as a peptidase inhibitor cocktail, 6.25 mg or 12.5 mg of rat brain membranes, various concentrations of LE or [HO-Tyr^1]-LE as competitor, and either 0.2 nM of [^{3}H]-diprenorphine, 1.65 nM of [^{3}H]-DAGO or 1 nM [^{3}H]-DPDPE as tracer, to assay for total opioid receptor, μ or δ sites, respectively, in 1 ml of 50 mM Tris-HCl buffer (pH 7.4). The tubes were incubated at room temperature with constant shaking for 1 hour. When using [^{3}H]-diprenorphine as tracer, the tubes were then centrifuged at 12,000g for 10 minutes to bring down the pellet. The pellets were washed three times with ice-cold 50 mM Tris-HCl buffer (without resuspending) and then were dissolved in 1 ml of Scintiverse II scintillation cocktail. When using [^{3}H]-DAGO or [^{3}H]-DPDPE as tracer, the rat brain membranes were separated from the supernatant and washed with ice-cold 50 mM Tris-HCl buffer by means of a rapid filtration technique. The filters retaining the rat brain membranes and bound tracer were placed in 4 ml of Scintiverse II scintillation cocktail and after being allowed to stand overnight, the radioactivity (CPM) in either the pellets or on the filters was determined using a Beckmann LS 5,000 TD scintillation counter (Fullerton, CA, USA). In a control experiment, HPLC analysis of the supernatant showed that both LE and [HO-Tyr^1]-LE were stable under radioreceptor assay conditions. Non-specific binding was determined in the presence of high concentrations of competitor (1 x 10^{-5} M). The IC_{50} values of LE and [HO-Tyr^1]-LE (concentration of competitor displacing 50% of bound tracer) were determined using the MINSQ non-linear curve-fitting program

(MicroMath Scientific software, Salt Lake City, UT, USA). The data were fitted to the following model:

$$B = T - \frac{T * C^N}{C^N + IC_{50}^N} + NS$$

Where: B = CPM in the presence of competitor
T = CPM in the absence of competitor
C = competitor concentration
N = slope factor
NS = CPM under non-specific binding conditions

Results

Chromatography

As in Chapters 3 and 4, leucine enkephalin and the products of the enzyme reaction were separated successfully from each other using the chromatographic system described above (Figure 5.2). In a control incubation where LE and ascorbic acid were injected into the system in concentrations equal to the initial concentrations of these reagents in the incubation mixture, LE had a retention time of 9.5 minutes and was well separated from ascorbic acid which eluted in a large solvent front at 1.4 minutes (Figure 5.2A). When the test incubation was applied to the system, two distinct additional peaks were seen on the chromatogram with retention times of 5.7 minutes and 7.3 minutes, respectively (Figure 5.2B). These peaks were collected and subjected to mass spectrometry as described earlier. An incubation time of one hour was chosen for our experiments as at this time point, most of the LE present was seen to have been consumed in the reaction and the levels of the major product were seen to plateau (Figure 5.1).

Ascorbic acid was included in the reaction mixture to prevent further oxidation of our hydroxylated products to the corresponding quinones.

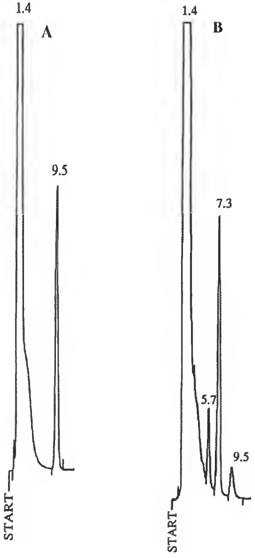


Figure 5.2. Chromatographs of control incubation (A) showing LE eluting after 9.5 minutes, distinct form ascorbic acid eluting in the solvent front after 1.4 minutes, and a test incubation after 30 minutes (B) showing the emergence of two new peaks with retention times of 5.7 and 7.3 minutes.

Mass spectrometry

ESI is a soft ionization technique that provides intact molecular ions from peptide solutions [Whitehouse et al. 1985]. However, selective fragmentation can also be effected by collision-induced dissociation (CID) in certain regions of the ion source [Allen and Vestal 1992, Katta et al. 1991, Smith et al. 1990]. The nomenclature scheme, as proposed by Roepstoff and Fohlman [Roepstorff and Fohlman 1984], used to label the sequence ions in the mass spectra of pentapeptides is shown in Figure 5.3.

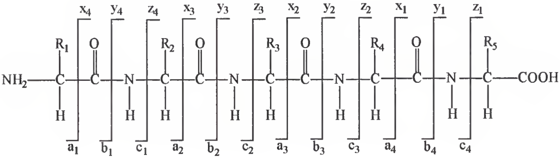


Figure 5.3. Nomenclature scheme for the labeling of sequence ions in the mass spectra of pentapeptides.

The ESI mass spectra obtained from LE are shown in Figure 5.4. Using soft ionization (repeller at 18 V, Figure 5.4A), only molecular ions ($[M+H]^+$, *m/z* 556, and $[M+Na]^+$, *m/z* 578) were observed. Although the formation of protonated molecules through acid-base equilibria in solution is preferred, the generation of sodiated species was unavoidable upon use of commercially available HPLC-grade solvents [Lehman 1982]. From the spectrum obtained under CID conditions (repeller at 50 V, Figure 5.4B), several important sequence ions (*m/z* 136, 221, 278, 397, and 425 for a_1, b_2, b_3, a_4, and b_4, respectively) directly derived from Figure 5.3 were assigned. Two intense internal fragments, $[a_4 \bullet y_2]_1$ at *m/z* 120 and $[a_4 \bullet y_3]_2$ at *m/z* 177, were also observed. These were the products of the dissociation of the protonated molecule (*m/z* 556). CID of the sodiated enkephalin was limited; only the loss of the C-terminal leucine moiety was prominent (*m/z* 465).

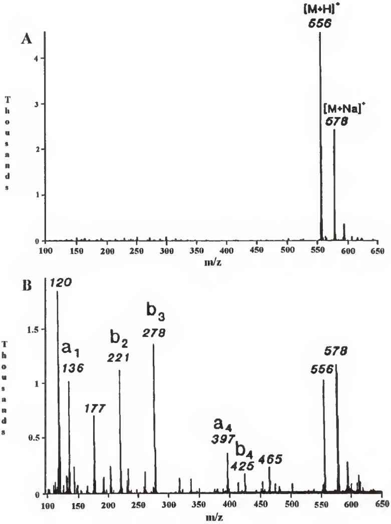


Figure 5.4. Electrospray ionization mass spectra of LE. (A) Repeller at 18 V; (B) Repeller at 50 V.

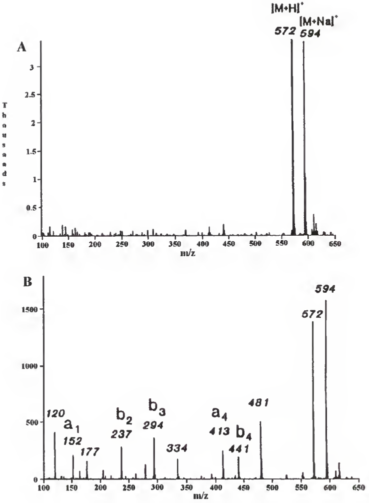


Figure 5.5. Electrospray ionization mass spectra of [HO-Tyr^1]-LE. (A) Repeller at 18 V; (B) Repeller at 50 V.

The identification of the hydroxylated products of LE was achieved by comparing the ESI mass spectra obtained to those of the parent peptide. The mono-hydroxylated enkephalin, [(HO)-Tyr^1]-LE, gave molecular ions at *m/z* 572 and 594 for $[M+H]^+$ and $[M+Na]^+$, respectively (Figure 5.5A). As shown in Figure 5.5B, a 16 Da increase was observed in the *m/z* value of the a and b sequence ions, starting with the a_1 ion of the series (*m/z* 152). On the other hand, the internal fragments *m/z* 120 and 177 were not affected. This is evidence that the phenylalanine moiety was not modified. Therefore, the oxygen was incorporated, as expected, into the [Tyr^1] residue. The ESI mass spectra recorded from the second product of the enzymatic reaction (Figure 5.6) indicated, based on the 32 Da increment of the molecular ions, and also of the a and b sequence ions from a_1 to a_4, *m/z* 168 to 429, as compared to the parent enkephalin, that additional hydroxylation also took place at the N-terminal tyrosine, to give a di-hydroxylated derivative, [(HO)$_2$-Tyr^1]-LE. The relative intensity of the peaks obtained from mass spectrometric analysis showed that [(HO)$_2$-Tyr^1]-LE was a minor product and therefore, sufficient quantities could not be collected for use in receptor binding studies.

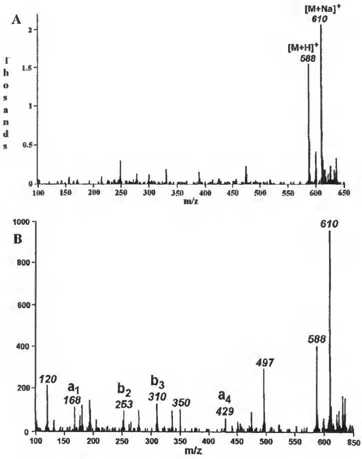


Figure 5.6. Electrospray ionization mass spectra of [(HO)$_2$-Tyr^1]-LE. (A) Repeller at 18 V; (B) Repeller at 50 V.

Receptor Binding Affinity

Representative displacement curves showing the binding of LE and [HO-Tyr^1]-LE to receptors in rat brain homogenate using [^{3}H]-diprenorphine, [^{3}H]-DAGO (μ sites) and [^{3}H]-DPDPE (δ sites) as tracer are shown in Figs 5.7, 5.8 and 5.9, respectively. The IC_{50} values obtained are shown in Table 5.1. The loss of affinity to total opioid receptors in rat brain homogenate observed when LE is hydroxylated by mushroom tyrosinase is mirrored by a loss of affinity to both μ and δ sites.

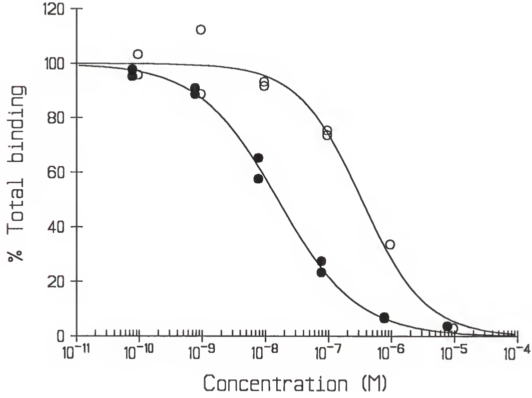


Figure 5.7. Displacement curves for LE (●) and [HO-Tyr^1]-LE (o) in rat brain homogenate using [^{3}H]-diprenorphine as tracer.

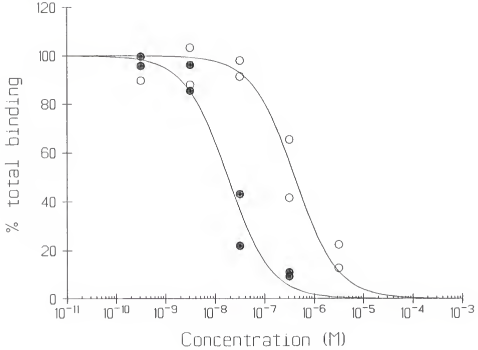


Figure 5.8. Displacement curves for LE (●) and [HO-Tyr^1]-LE (o) in rat brain homogenate using [^{3}H]-DAGO as tracer.

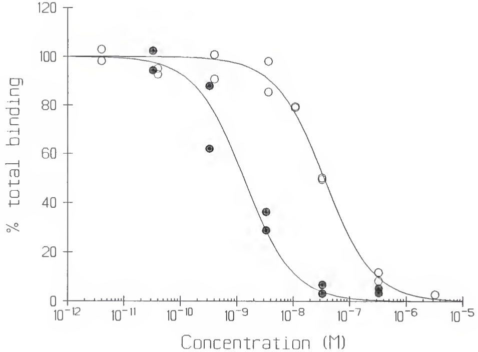


Figure 5.9. Displacement curves for LE (●) and [HO-Tyr^1]-LE (o) in rat brain homogenate using [^{3}H]-DPDPE as tracer.

Table 5.1. Summary of IC_{50} values obtained in rat brain homogenate receptor binding studies using [^{3}H]-diprenorphine, [^{3}H]-DAGO and [^{3}H]-DPDPE as tracers

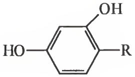
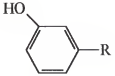
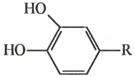
	IC_{50} (nM) ± SD, n		
	[^{3}H]-diprenorphine	[^{3}H]-DAGO	[^{3}H]-DPDPE
LE	26 ± 11, 4	18 ± 4, 3	1.2 ± 0.25, 3
[HO-Tyr^1]-LE	440 ± 150, 4	381 ± 106, 3	34 ± 2, 3

Discussion

Using mass spectrometry, the products of the reaction between leucine enkephalin and mushroom tyrosinase in the presence of ascorbic acid were positively identified as [HO-Tyr^1]-LE and [$(HO)_2$-Tyr^1]-LE. The identification of [HO-Tyr^1]-LE confirms the findings of previous workers [Rosei et al. 1991, Rosei et al. 1989], however, the dihydroxylated derivative, [$(HO)_2$-Tyr^1]-LE, was not isolated by these investigators as its presence probably could not be detected by means of ultraviolet spectrometry without the benefit of separation of reaction components by HPLC. Tyrosinase has been shown to produce 5-OH-dopa from dopa in the presence of ascorbic acid [Hansson et al. 1981]. Similarly, [HO-Tyr^1]-LE seems to act as a substrate for tyrosinase to give [$(HO)_2$-Tyr^1]-LE. This stepwise reaction mechanism is supported by the slower and delayed formation of the dihydroxylated derivative compared to the formation of [$(HO)_2$-Tyr^1]-LE.

The biological activity of opioid peptides with modified Tyr^1 residues has not been studied to the same extent as [des-Tyr^1] opioid peptides which generally show a loss of activity [Hansen and Morgan 1984, Shimohigashi 1986] (Table 5.2). Hansen et al. [Hansen et al. 1985] showed that the introduction of methyl groups in the ortho position

Table 5.2. Effect of modification of Tyr^1 moiety of opioid peptides on opioid binding affinity.

Modification at Tyr^1	Effect	Reference
[des-Tyr^1] opioid peptide	loss of opioid binding affinity	Hansen and Morgan 1984 Shimohigashi 1986
	increased opioid binding affinity	Hansen et al. 1985
	reduced opioid binding affinity	Judd et al. 1985
	reduced opioid binding affinity	This study

R= opioid peptide analog

of the Tyr^1 ring of enkephalin analogs greatly increased opioid binding affinity. By contrast, Judd et al. [Judd et al. 1985] demonstrated that D-Ala^2 methionine enkephalin amide analogs with a hydroxy group in the meta position rather than the para position of the Tyr^1 ring showed reduced affinity to μ_1, μ_2, δ and κ opioid receptors. In our studies, a decrease in opioid binding affinity to both μ and δ receptors was observed when a hydroxy group was introduced at the meta position of the Tyr^1 ring of LE, in addition to the hydroxy group already present at the para position. We presumed that the first hydroxylation took place at the meta position as the hydroxy group in the para position acts as a driving force for the electrophilic substitution. Therefore, in conjunction with the observations of Judd et al. [Judd et al. 1985], our findings indicate that the presence of a hydroxy group in the meta position rather than the absence of one in the para position gives rise to a decrease in opioid receptor binding in enkephalins.

Tyrosinase and enkephalin immunoreactivities have been identified in isolated cells [Kimura et al. 1992] and the spinal and brain regions in some species, namely the locus coeruleus complex in the cat [Zhuo et al. 1992] and the ventral tegmental area in the rat [Sesack and Pickel 1992]. Although aminopeptidases, carboxypeptidases and enkephalinases are thought to be mainly responsible for the metabolic deactivation of LE [Venturelli et al. 1985], given that tyrosinase and LE have been found to co-exist in vivo, and that the product of the reaction between these two entities shows decreased affinity to both μ and δ opioid receptors compared to the parent enkephalin, we speculate that tyrosinase may also contribute to the metabolic fate of LE in vivo.

CHAPTER 6
CONCLUSIONS

In the work carried out for this dissertation, the feasibilty of both an enzyme-linked immunosorbent assay (ELISA) and an homogeneous fluorescence immunoassay for leucine enkephalin (LE) was evaluated. Two high-performance liquid chromatography (HPLC) approaches for opioid peptides using LE as a model peptide and enzymatic derivatization by tyrosinase were also evaluated. One of these HPLC approaches used electrochemical detection as a means of quantitation and the other used fluorescence detection following a second fluorogenic derivatization step with 1,2-diamino-1,2-diphenylethane (DPE). In addition, since hydroxylated products were produced from the reaction between tyrosinase and leucine enkephalin in the development of the HPLC approaches, and since tyrosinase and enkephalins have been found to co-exist in vivo, the identity of these products was determined by mass spectrometry and their biological activity in rat brain homogenate was investigated.

The formation of a sandwich between an anti-LE antibody, a biotinylated LE derivative and avidin conjugated to an enzyme was a requirement for the successful development of the ELISA proposed for this dissertation since in this assay, LE and biotinylated LE should compete for antibody binding sites and antibody-bound biotinylated LE should subsequently be detected through the use of enzyme-labeled avidin on addition of the enzyme substrate. The formation of a sandwich could not be achieved using either the antibody produced in this laboratory or the commercial antiserum tested and any of the biotinylated derivatives synthesized. Therefore, a workable ELISA for LE could not be developed using the proposed approach. ELISAs for β-endorphin and dynorphin A1-13 have already been developed using the same strategy proposed here for the development of an ELISA for LE [Hochhaus and Sadee 1988, Hochhaus and Hu 1990]. However, these opioid peptides consist of longer amino acid chains than LE (31 amino acids for β-endorphin and 13 amino acids for dynorphin compared to only 5 amino acids for LE) and therefore, it is suggested that if a biotinylated LE derivative including a longer spacer arm can be synthesized, a sandwich between anti-LE antibody, this biotinylated LE derivative and avidin could be formed, leading to the successful development of an ELISA for LE based on the same principles used in the ELISAs for β-endorphin and dynorphin A1-13 which have been described earlier [Hochhaus and Sadee 1988, Hochhaus and Hu 1990].

The successful development of the homogeneous fluorescence immunoassay proposed in this dissertation relied on a lack of sandwich formation between an anti-LE antibody, a biotinylated derivative and fluorescence-labeled avidin since here, LE and biotinylated LE compete for antibody binding sites, and subsequently, free biotinylated LE is detected on addition of fluorescence-labeled avidin due to the increase in fluorescence observed on interaction between biotinylated LE and fluorescence-labeled avidin. A lack of sandwich formation was seen using the anti-LE antibody produced in this laboratory, a biotinylated derivative biotinylated at the N-terminal end of the peptide without a spacer arm (BLE) and fluorescein isothiocyanate-labeled avidin (FITC-avidin). Although as expected, an increase in fluorescence was seen in our homogenous fluorescence immunoassay when increased amounts of LE were present in the sample, we observed that at higher concentrations of LE ($1*10^{-8}$ moles/ml and above), the analyte interacted directly with FITC-avidin to produce an increase in fluorescence in the absence of BLE. [des-Tyr^1]-LE and pentaglycine at the same concentrations did not produce an increase in fluorescence in the presence of FITC-avidin, suggesting that the effect observed in the homogeneous immunoassay at higher concentrations of LE was due to an interaction between the tyrosine moiety of LE and the fluorescein groups of FITC-avidin. The use of an antibody with higher affinity for LE may allow the successful development of this type of homogenous fluorescence immunoassay for LE since less LE could be used in the assay and therefore the direct interaction observed at high concentrations between LE and FITC-avidin would be avoided. The use of laser-induced fluorescence may also contribute to the succesful development of this type of assay since less FITC-avidin would be required to produce a measurable signal on interaction with smaller quantities of BLE. As a consequence, less LE would be required to displace sufficient BLE to produce a signal on interaction with FITC-avidin and therefore, once again, high concentrations of LE which interfere with the success of the assay would be avoided.

Two HPLC approaches for the determination opioid peptides were developed for this dissertation, one with electrochemical detection and the other with fluorescence detection. Both of these approaches involved the specific enzymatic derivatization of the Tyr^1 moiety of the model peptide LE by mushroom tyrosinase. This enzymatic derivatization selectively introduced an hydroxy group to the Tyr^1 moiety of the peptide, thus presenting several analytical advantages. Firstly, the hydroxylation of LE rendered it more easily oxidizable than the parent peptide, thus allowing electrochemical detection at much lower oxidation potentials and therefore avoiding many of the disadvantages associated with the use of high oxidation potentials such as reduced selectivity, high background current, baseline drift and baseline noise. Secondly, the production of a catechol permitted the selective clean-up of the analyte using boronate gels. Thirdly, controlled oxidation of the hydroxylated derivative to give the corresponding quinone enabled the use a secondary fluorogenic condensation reaction with DPE and subsequent quantification the analyte by fluorescence detection.

The HPLC-ED and HPLC-FL approaches described in Chapters 3 and 4 yielded limits of detection for LE in buffer samples of 170 fmol/inj and 500 fmol/inj, respectively and 360 fmol/inj and 500 fmol/inj for LE in CSF, respectively. These limits of detection compared favorably to existing HPLC assays for opioid peptides with on-line detection. The limits of detection for LE in CSF corresponded to 8.8 pmol/ml for the HPLC-ED assay and 12 pmol/ml for the HPLC-FL assay. Endogenous levels of opioid peptides in human CSF lie in the fmol/ml range and therefore, in their present state of development, the HPLC approaches described here are inadequate for such determinations. However, given sufficient sample (>1 ml), these approaches may be suitable for the determination of elevated physiological levels to be expected in clinical studies.

The HPLC-ED approach described in Chapter 3 involved a single 5 minute derivatization step prior to injection of the sample into the HPLC system compared to two one hour derivatization steps for the HPLC-FL assay described in Chapter 4. The HPLC-ED method also resulted in a slightly lower limit of detection for LE than the HPLC-FL method (170 fmol/inj in buffer and 360 fmol/inj in CSF compared to 500 fmol/inj in both buffer and CSF) and yields a cleaner chromatograph with the peak of interest eluting after a shorter retention time (6 minutes compared to 22.5 minutes). Therefore, of the two HPLC approaches described in this dissertation, based on sensitivity, convenience and practicality, the HPLC-ED assay is the method of choice for the analysis of opioid peptides in CSF. It is proposed that increased sensitivity could be achieved using the HPLC-FL approach by incorporating microbore HPLC and laser-induced fluorescence into the procedure.

Hydroxylated derivatives of LE were produced using mushroom tyrosinase in the HPLC assays for opioid peptides described in Chapters 3 and 4. In Chapter 5 the structure of the major product of the reaction between LE and mushroom tyrosinase was determined by electrospray ionization mass spectrometry to be [HO-Tyr^1]-LE and the minor product of the reaction was identified as [$(HO)_2$-Tyr^1]-LE. The affinity of [HO-Tyr^1]-LE to opioid receptors in rat brain homogenate was determined by radioreceptor assay. Hydroxylation of LE was found to decrease receptor affinity to both μ and δ opioid receptor sites by a factor of about 20. Tyrosinase and enkephalin have been found to co-exist in isolated cells and in the spinal and brain regions of some species. Therefore, since we have demonstrated that the product of the reaction between these two entities shows decreased affinity to opioid receptors compared to the parent enkephalin, we speculate that tyrosinase may play a role in the metabolic pathway of LE in vivo.

APPENDIX A
DATA FOR HPLC-ED APPROACH

Curve 1

Tyrosinase derivatization, boronate clean up
Matrix: Buffer
Equation: $y = 27611x + 2288$, $r^2 = 0.9977$

LE mol/inj	Peak area
0	0
$5.33*10^{-13}$	17597
$1.07*10^{-12}$	32566
$2.13*10^{-12}$	64947
$3.20*10^{-12}$	87745
$4.80*10^{-12}$	134842

Curve 2

Tyrosinase derivatization, boronate clean up
Matrix: Buffer
Equation: $y = 30648x - 1752$, $r^2 = 0.9876$

LE mol/inj	Peak area
0	0
$3.37*10^{-13}$	5077
$5.06*10^{-13}$	15565
$8.44*10^{-13}$	17240
$1.18*10^{-12}$	40429
$1.69*10^{-12}$	52711
$2.53*10^{-12}$	74861
$3.37*10^{-12}$	100722

Curve 3

Tyrosinase derivatization, boronate clean up
Matrix: Buffer
Equation: $y = 25953x + 3341$, $r^2 = 0.9922$

LE mol/inj	Peak height (mm)
0	0
$1.68*10^{-13}$	9573
$3.37*10^{-13}$	14456
$5.06*10^{-13}$	15197
$1.69*10^{-12}$	48918
$2.53*10^{-12}$	67658

Curve 4

Tyrosinase reaction, boronate clean up
Matrix: CSF
Equation: $y = 21753x - 12089$, $r^2 = 0.9957$

LE mol/inj	Peak area
0	11724
$3.60*10^{-13}$	11205
$7.22*10^{-13}$	30931
$1.42*10^{-12}$	43348
$2.73*10^{-12}$	74283
$4.29*10^{-12}$	109291
$7.01*10^{-12}$	169232
$9.63*10^{-12}$	215784

Curve 5

Tyrosinase reaction, boronate clean up
Matrix: CSF
Equation: $y = 21179x - 16355$, $r^2 = 0.9904$

LE mol/inj	Peak area
0	21412
$3.60*10^{-13}$	7743†
$7.22*10^{-13}$	29865
$1.42*10^{-12}$	42426
$2.73*10^{-12}$	65917
$4.29*10^{-12}$	111943
$7.01*10^{-12}$	176539
$9.63*10^{-12}$	212826

†outlier, calculated concentration deviates from nominal concentration by >20% (H. T. Karnes, personal communication)

Curve 6

Tyrosinase reaction, boronate clean up
Matrix: CSF
Equation: $y = 23240x - 10701$, $r^2 = 0.9971$

LE mol/inj	Peak area
0	10509
$3.60*10^{-13}$	15452
$7.22*10^{-13}$	28020
$1.42*10^{-12}$	44913
$2.73*10^{-12}$	73904
$4.29*10^{-12}$	116036
$7.01*10^{-12}$	170143

Curve 7

No derivatization, no boronate clean up
Matrix: CSF
Equation: $y = 10319x - 44657$, $r^2 = 0.9770$

LE mol/inj	Peak area
0	0
$8.82*10^{-12}$	93356
$17.28*10^{-12}$	113333
$25.56*10^{-12}$	156482
$33.30*10^{-12}$	230196
$43.2*10^{-12}$	422738
$69.29*10^{-12}$	714440
$85.67*10^{-12}$	841176

Curve 8

No derivatization, no boronate clean up
Matrix: CSF
Equation: $y = 9006x - 10275$, $r^2 = 0.9570$

LE mol/inj	Peak area
0	0
$17.28*10^{-12}$	149435
$25.56*10^{-12}$	217441
$33.30*10^{-12}$	193110
$43.2*10^{-12}$	463885
$69.29*10^{-12}$	660135
$85.67*10^{-12}$	720965

Curve 9

No derivatization, no boronate clean up
Matrix: CSF
Equation: $y = 11326x + 59177$, $r^2 = 0.9529$

LE mol/inj	Peak area
0	0
$8.82*10^{-12}$	259029
$17.28*10^{-12}$	240843†
$25.56*10^{-12}$	265387
$33.30*10^{-12}$	231334†
$43.2*10^{-12}$	646006
$69.29*10^{-12}$	762035
$85.67*10^{-12}$	1064494

†outlier

APPENDIX B
DATA FOR HPLC-FL APPROACH

Curve 1

Non-concentrated reagents
Matrix: Buffer
Equation: $y = 1.79*10^{12}x + 0.4064$, $r^2 = 0.9966$

LE mol/inj	Peak area
0	0
$5.62*10^{-13}$	1.5338
$1.12*10^{-12}$	2.7748
$2.23*10^{-12}$	4.0617
$4.38*10^{-12}$	8.5962
$8.76*10^{-12}$	15.9292

Curve 2

Non-concentrated reagents
Matrix: buffer
Equation: $y = 1.14*10^{13}x + 2.2749$, $r^2 = 0.9836$

LE mol/inj	Peak height (mm)
0	0
$8.91*10^{-13}$	13
$1.80*10^{-12}$	26
$3.59*10^{-12}$	39
$5.39*10^{-12}$	70
$7.18*10^{-12}$	81

Curve 3

Non-concentrated reagents
Matrix: buffer
Equation: $y = 3.40*10^{13}x - 1.3522$, $r^2 = 0.9971$

LE mol/inj	Peak height (mm)
0	0
$2.81*10^{-13}$	9
$3.65*10^{-13}$	lost
$5.62*10^{-13}$	15
$1.12*10^{-12}$	37
$2.23*10^{-12}$	75

Curve 4

Non-concentrated reagents
Matrix: CSF
Equation: $y = 3.89*10^{12}x - 0.6900$, $r^2 = 0.9975$

LE mol/inj	Peak area
0	0
$4.97*10^{-13}$	0.6753
$8.86*10^{-13}$	2.7875
$2.12*10^{-12}$	7.7407
$3.96*10^{-12}$	14.0380
$7.24*10^{-12}$	27.8690

Curve 5

Non-concentrated reagents
Matrix: CSF
Equation: $y = 1.08*10^{13}x - 0.7695$, $r^2 = 0.9670$

LE mol/inj	Peak height (mm)
0	0
$8.91*10^{-13}$	12
$1.78*10^{-12}$	17
$5.38*10^{-12}$	48
$7.18*10^{-12}$	84

Curve 6

Concentrated reagents
Matrix: Buffer
Equation: $y = 2.25*10^{12}x - 0.8243$, $r^2 = 0.9926$

LE mol/inj	Peak area
0	0
$4.47*10^{-13}$	1.14
$1.79*10^{-12}$	7.151
$6.68*10^{-12}$	14.916
$1.67*10^{-12}$	38.496

Curve 7

Concentrated reagents
Matrix: Buffer
Equation: $y = 5.09*10^{12}x - 0.8542$, $r^2 = 0.9984$

LE mol/inj	Peak area
0	0
$5.01*10^{-13}$	1.1262
$1*10^{-12}$	4.1923
$2*10^{-12}$	8.9492
$3*10^{-12}$	14.35
$4.67*10^{-12}$	28.442†
$6.68*10^{-12}$	33.319

†outlier, calculated concentration deviates from nominal concentration by >20% (H. T. Karnes, personal communication)

Curve 8

Concentrated reagents
Matrix: CSF
Equation: $y = 9.91*10^{11}x + 0.5776$, $r^2 = 0.9925$

LE mol/inj	Peak area
0	0.79768
$5*10^{-13}$	1.0174
$1*10^{-12}$	1.3522
$2*10^{-12}$	lost
$3*10^{-12}$	5.2685
$4.68*10^{-12}$	3.0910†

†outlier

Curve 9

Concentrated reagents
Matrix: CSF
Equation: $y = 1.76*10^{12}x + 3.0077$, $r^2 = 0.9853$

LE mol/inj	Peak area
0	2.2941
$4.69*10^{-13}$	3.5183
$8.85*10^{-13}$	2.4436†
$1.95*10^{-12}$	7.0437
$2.88*10^{-12}$	3.7482†
$4.41*10^{-12}$	10.505

†outlier

Curve 10

Concentrated reagents
Matrix: CSF
Equation: $y = 3.45*10^{12}x + 1.0393$, $r^2 = 0.9975$

LE mol/inj	Peak area
0	1.4506
$4.69*10^{-13}$	2.6145
$8.85*10^{-13}$	4.3134
$1.95*10^{-12}$	4.0743†
$2.88*10^{-12}$	9.3891
$4.41*10^{-12}$	17.243

†outlier

Curve 11

Concentrated reagents, 5 minute tyrosinase reaction
Matrix: Buffer
Equation: $y = 7.67*10^{12}x + 4.9244$, $r^2 = 0.9922$

LE mol/inj	Peak height (mm)
0	0
$2.77*10^{-13}$	10.5
$5.54*10^{-13}$	11.5
$1.38*10^{-12}$	18
$2.77*10^{-12}$	24
$5.54*10^{-12}$	44.2
$8.31*10^{-12}$	64.5
$1.38*10^{-11}$	115

Curve 12

Concentrated reagents, 5 minute tyrosinase reaction
Matrix: Buffer
Equation: $y = 2.50*10^{12}x + 4.449$, $r^2 = 0.9779$

LE mol/inj	Peak height (mm)
0	3.5
$2.77*10^{-13}$	4.5
$5.54*10^{-13}$	7
$1.38*10^{-12}$	8.5
$2.77*10^{-12}$	11.5
$5.54*10^{-12}$	18

Curve 13

Concentrated reagents, 5 minute tyrosinase reaction
Matrix: Buffer
Equation: $y = 9.91*10^{12}x + 6.4066$, $r^2 = 0.9985$

LE mol/inj	Peak height (mm)
0	6
$2.77*10^{-13}$	10.5
$5.54*10^{-13}$	13
$1.38*10^{-12}$	16.5
$2.77*10^{-12}$	11†
$5.54*10^{-12}$	63.5
$6.64*10^{-12}$	43.5†
$1.11*10^{-11}$	83†
$1.38*10^{-11}$	143

†outlier

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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